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Stephanie Leila Parsley

The Effects of Environmental Enrichment in the CaMKII^{T286A} Mutant Mouse

Submitted for the degree of

Doctor of Philosophy

University College London

(October 2000 – August 2005)

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Abstract

The activity-dependent plasticity of synaptic strengths is believed to underlie learning and memory in the brain. One experimental form of synaptic plasticity is long-term potentiation (LTP), a phenomena that requires the actions of the highly abundant Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII). In support of the hypothesis that an LTP-like process may exist physiologically and be important for cognitive processing, hippocampal-dependent learning and memory are severely impaired in transgenic mice that possess a point-mutation (T286A) in the α-isoform of CaMKII that blocks the ability of the kinase to autophosphorylate at residue Thr286 and exhibit Ca^{2+}-independent activity.

I have studied the properties of excitatory synaptic transmission in acute hippocampal brain slices from adult $\alpha CaMKII^{T286A}$ mutant mice and compared them to those from wild-type animals in order to investigate the consequences that may arise from the loss of CaMKII-dependent endogenous forms of synaptic plasticity. Surprisingly, no differences in the properties of evoked CA1 synaptic responses were found, suggesting that the loss of CaMKII function does not affect synaptic connectivity in these animals.

To promote physiological brain plasticity and therefore the potential for detecting differences between the genotypes, mice were also raised in environmentally-enriched housing conditions. 3-5 weeks of enrichment did not affect the properties of evoked synaptic transmission in wild-type CA1 neurones and gross levels of excitatory input, assessed by median mEPSC amplitudes and measures of dendritic spine densities, were also unaffected. These neuronal properties were all significantly altered however, following environmental enrichment in the mutant animals. Thus, Thr286 autophosphorylation-independent forms of plasticity exist *in vivo* and are recruited by enriched experience in the mutant mouse. One explanation why such changes were not similarly observed in the wild-types may be that they are actively reversed by Thr286 autophosphorylation-dependent processes. In addition to impairing LTP, the T286A mutation may also impair homeostatic processes that act to maintain the properties of excitatory synaptic transmission in the mouse hippocampus.

Dedication

This thesis is dedicated to my loving family and friends

&

to silver linings.

Acknowledgments

I acknowledge and thank my academic supervisor, Dr. Frances Edwards, for her supervision and support during my time in her laboratory. I also thank The Wellcome Trust and the Neuroscience Ph.D. programme within the Physiology Department at U.C.L. for providing me with the prized opportunity to undertake this research.

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An enormous hug and thanks also go to Joyce (the best flat-mate ever) and to Stuart. Thanks for being my London oasis, for the ears, the advice on so many matters and the constant flow of G&Ts.

Finally, to Mauro – my motivation; the F.B.T is finally finished!!!

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List of Abreviations

7ClK	7-chlorokynurenic acid
A	Alanine
ABC	avidin-biotin enzyme complex
AC	Adenylyl cyclase
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BDNF	Brain derived neurotrophic factor
CA	*Cornu ammonis*
Ca^{2+}/CaM	Ca^{2+}-bound CaM
CaM	Calmodulin
CaMKII	Ca^{2+}/Calmodulin-dependent protein kinase
cAMP	Cyclic adenosine monophosphate
CREB	cAMP response element-binding protein
DAB	Diaminobenzidine
DMSO	Dimethylsulphoxide
EPSC	Excitatory postsynaptic current
EPSP	Excitatory postsynaptic potential
F-actin	Filamentous actin
FRET	Fluorescence resonance energy transfer
GABA	γ-amino butyric acid
G-actin	Globular actin
GFP	Green fluorescent protein

HEK	Human embryonic kidney
ISI	Inter-stimulus interval
Kd	Dissociation equilibrium constant
LTD	Long-term depression
LTP	Long-term potentiation
mEPSC	Miniature excitatory postsynaptic current
mGluR	Metabotropic glutamate receptor
mRNA	Messenger Ribonucleic Acid
NBQX	QX-314,2.3-dioxo-6-nitro-1,2,3,4-tetrahydro-quinoxaline-7-suphonamide
NSF	N-ethylmaleimide-sensitive fusion protein
P	Postnatal day
PB	Phosphate buffer
PCR	Polymerase chain reaction
PDZ	Post-synaptic density 95/disc large/zonula occludens-1
PFA	Paraformaldehyde
PKA	cAMP-dependent protein kinase
PKC	Protein kinase C
PP	Protein phosphatase
PPF	Paired-pulse facilitation
PPR	Paired-pulse ratio
PSD	Postsynaptic density
s.d.	Standard deviation
T or Thr	Threonine
TTX	Tetrodotoxin

CHAPTER 1
Introduction

1.1 Thesis Rationale & Aims

The aim of the experiments contained within this thesis was to evolve further our current understanding about the plastic nature of synaptic connections within the brain.

The modification of the strength and number of synaptic connections between neurones in the nervous system is believed to be a fundamental process for brain function. Indeed the phenomena that encompass synaptic plasticity are thought to be crucial for the refinement of functional circuits in various brain regions during development (e.g. Goodman and Shatz, 1993) as well as being important for the recovery from brain injury and may even underlie various neurological conditions such as drug addiction (e.g. Kolb, 2003;Wolf et al., 2004). Furthermore, the plasticity of synaptic efficacy is thought to be one way through which the brain may process cognitive information and provide a mechanism by which the brain is able to learn and form memories (Martin et al., 2000;Martin and Morris, 2002;Lynch, 2004). The long-term potentiation (LTP) and long-term depression (LTD) of synaptic strengths, the respective activity-dependent strengthening and weakening of synapses, are two well-studied paradigms of synaptic plasticity that can be experimentally induced using either chemical or electrical stimulation. These phenomena have been identified in many regions of the nervous system, including the neurones of the hippocampus, numerous regions of the cerebral cortex, the cerebellum, striatum, amygdala, and the spinal cord (Kemp and Bashir, 2001;Lynch, 2004). However, it is the plasticity of synaptic connections within the hippocampus that has received the most intense experimental investigation. This is due to the unanimous recognition that the hippocampus plays a crucial role in the formation of long-term memories in humans (Squire, 1992).

A wealth of experimental data has correlated impairments in the ability to induce LTP (brought about, for instance, through pharmacological or genetic manipulation) with disrupted learning and memory abilities and this has made a strong case for the

proposal that LTP-like mechanisms also occur *in vivo* and are important for learning and memory formation in the brain (e.g. Morris et al., 1986;McNaughton et al., 1986;Giese et al., 1998;Rotenberg et al., 2000;Jones et al., 2001a;Bejar et al., 2002). This has been further supported by the demonstration that differences in the robustness of LTP expression between different strains of inbred mice correlate with differences in their abilities to perform in various behavioural tests of learning and memory (Nguyen et al., 2000;Jones et al., 2001b;Schimanski and Nguyen, 2004). Moreover, saturating synaptic efficacy at hippocampal synapses, using LTP-inducing stimuli *in vivo*, has been found to interfere with the subsequent ability of animals to learn hippocampal-dependent spatial tasks (McNaughton et al., 1986;Castro et al., 1989;Barnes et al., 1994;Moser et al., 1998;Moser and Moser, 1999) supporting the consensus that synaptic potentiation is required for learning. It must also be noted that although LTD has received less interest for its potential role as a cellular mechanism of learning in the hippocampus, it also stands as a theoretically valid model and may underlie some forms of learning and memory (Willshaw and Dayan, 1990;Paulsen and Sejnowski, 2000;Braunewell and Manahan-Vaughan, 2001); this has been particularly well demonstrated in the cerebellum, where the role of LTD in cerebellar-dependent motor learning is well accepted (Ito, 2001;Boyden et al., 2004). Recent work in the hippocampus has also suggested that LTD may be specifically important for novelty acquisition (Kemp and Manahan-Vaughan, 2004) and for episodic-like flexible memory of new and changing information (Zeng et al., 2001). Thus the most likely scenario is that the potentiation and depression of specific hippocampal synapses occurs in parallel and acts in concert to equip the hippocampus with the appropriate information-coding strategies that can fulfill the various functions of the hippocampal circuitry. This proposal received recent support from a study that correlated changes in the ability to induce either LTP or LTD in the hippocampi of behaving rats with specific environmental conditions that required the acquisition of different types of new information (Kemp and Manahan-Vaughan, 2004).

If these experimental models of synaptic plasticity also occur *in vivo*, in response to natural physiological stimuli, and characterise the processes recruited during experience-dependent hippocampal functioning, then it should be possible to detect evidence that such processes have occurred in a wild-type mouse brain by comparing

the properties of synaptic efficacy with those from a mouse where the underlying molecular mechanisms required for synaptic plasticity, learning and memory have been perturbed. If differences do exist, they could give some clues to the *in vivo* characteristics of endogenous synaptic plasticity in the hippocampus. This rationale forms the basis of the investigations reported within this thesis.

To investigate this proposal, synaptic and morphological properties of CA1 pyramidal cells in the hippocampus were studied in wild-type mice where all *in vivo* plastic processes remain intact and compared to those from a transgenic mouse where LTP at CA1 excitatory synapses and hippocampal-dependent learning are severely impaired. The transgenic chosen has a mutation that blocks the function of a key protein required for LTP expression at CA1 synapses. This targeted protein is the α-subunit of a multi-functional threonine-serine protein kinase, Ca^{2+}/calmodulin-dependent protein kinase II (αCaMKII). Its function is impaired due to the substitution of the amino acid at position 286, threonine 286 (Thr286), with alanine (Giese et al., 1998). This substitution does not impair the initial activation or kinase activity of αCaMKII but it specifically blocks its ability to autophosphorylate at residue Thr286, a process that is required for the kinase to exhibit "autonomous" kinase activity – the ability of the kinase to remain active after its initial activating trigger (a rise in Ca^{2+} concentration) is no longer present (Hanson et al., 1989). LTP at CA1 inputs and hippocampal-dependent learning and memory are both severely impaired in the $\alpha CaMKII^{T286A}$ homozygote mutant mice (Giese et al., 1998;Need and Giese, 2003;Irvine et al., 2005) rendering it a suitable model for this study.

The motive for choosing this particular transgenic mouse was that despite the many questions and controversies that remain regarding the underlying mechanisms of LTP, a diverse range of pharmacological/biochemical, genetic and theoretical investigations have all yielded evidence that supports the central hypothesis that this highly abundant synaptic kinase is a key and necessary component for the induction of hippocampal LTP in adult mice. For example, the injection of specific inhibitors of CaMKII into postsynaptic cells blocks LTP induction indicating that CaMKII is necessary for LTP (Malenka et al., 1989;Malinow et al., 1989). Moreover, the introduction of constitutively active αCaMKII into cells is sufficient by itself to induce and indeed

occlude further LTP, supporting the hypothesis that the mechanisms by which CaMKII and LTP induction potentiate synaptic transmission are the same (Pettit et al., 1994;Lledo et al., 1995). Furthermore, both the αCaMKII knock-out and T286A transgenic mice show severely impaired LTP and deficits in behavioural tests of hippocampal-dependent learning (Silva et al., 1992a;Silva et al., 1992b;Giese et al., 1998;Need and Giese, 2003;Irvine et al., 2005). The role of CaMKII in long-lasting synaptic potentiation has also been supported by theoretical work that has considered the biochemical properties and regulation of CaMKII activation and inactivation, the control of its synaptic location and the interactions it makes with other synaptic molecules (Zhabotinsky, 2000;Lisman and Zhabotinsky, 2001;Lisman et al., 2002;Bradshaw et al., 2003).

An important element of this thesis is that unlike the majority of studies that are concerned with understanding plasticity in the brain, the aim of these experiments was not to investigate the plastic *capabilities* of neurones in response to exogenous forms stimulation, but it was to uncover evidence of endogenous brain plasticity by comparing the properties of hippocampal excitatory connectivity in wild-type and $\alpha CaMKII^{T286A}$ mice, and thus provide important evidence that CaMKII-dependent plasticity is indeed a physiological phenomenon that takes place in the behaving animal.

Over the past decades, a vast number of experiments have been performed documenting the plastic capabilities of neurones, but showing how the properties of neurones <u>can</u> be altered in response to specific patterns of experimental stimulation may not necessarily reflect how neurones <u>do</u> respond and change when faced with *in vivo* forms of neural activity. The aim of this thesis was thus to uncover evidence of endogenous plasticity in order to understand better the role and form of synaptic plasticity *in vivo*.

Moreover, the technique of enriching the animals' living conditions was employed in this thesis in order to promote *in vivo* hippocampal function and synaptic activity. The hypothesis was that any changes in CA1 neuronal properties that could be detected as a result of enrichment-mediated *in vivo* synaptic plasticity would be different between wild-type and mutant mice. The environmentally enriched cages were bigger than the standard laboratory cages that housed control (non-enriched) wild-type and mutant

animals and contained a large selection of novel objects and bedding materials. The contents and arrangement of these cages were changed at least 5 days per week for 3-5 weeks. The aim of enrichment was to increase the animals' exposure to environmental novelty and thus enhance the opportunity that the mice had to employ hippocampal-dependent processes, like spatial navigation and learning (O'Keefe, 1999;Jeffery and Hayman, 2004). The enriched animals also had the opportunity and incentive to be more physically active than the mice in standard cages. Increased levels of voluntary exercise in rodents and the exposure to enriched environments have both been shown to result in changes in brain biochemistry, structure and physiology that are suggestive of enhanced levels of plasticity having taken place and enhanced plastic capabilities of the brain (Rosenzweig and Bennett, 1996;Van Praag et al., 2000;Mohammed et al., 2002;Cotman and Berchtold, 2002). These studies support the rationale for using environmental enrichment to study the consequences that arise from the lack of CaMKII-dependent synaptic plasticity in αCaMKII^{T286A} mutant mice compared to wild-type controls.

The aim of the following introduction sections is to present an overview of some of the issues regarding the physiology of synaptic plasticity in the hippocampus and its relationship to hippocampal functions *in vivo*. A description of the known molecular properties of CaMKII will also be presented along with a discussion of how these properties are thought to enable this molecule to translate Ca^{2+} signals into long-lasting changes in synaptic function.

1.2 The Hippocampus & Declarative Memory

Declarative memory (also referred to as explicit memory) is the conscious recollection of facts and autobiographical events. In contrast, non-declarative (or implicit) forms of memory, that are tested using performance-based criteria, like procedural motor memory, priming, classical conditioning, non-associative habituation and sensitisation, do not require *conscious* recall and depend upon anatomically distinct memory systems within the brain (reviewed in Zola-Morgan and Squire, 1993).

In the 1950's the hypothesised role of the hippocampus in long-term declarative memory formation became firmly consolidated following the finding that surgical removal of the medial temporal lobes in human patients (a procedure then carried out to

treat severe epilepsy) resulted in the precipitation of severe anterograde amnesia and a level of retrograde amnesia (the respective inability to form new long-term memories and recall previously formed memories; Scoville and Milner, 1957;Penfield and Milner, 1958). These studies also resolved several other unknown questions about the brain processes that underlie learning in the brain. They showed that short-term working memory as well as non-declarative forms of learning did not require the structures within lay within the bisected medial temporal lobes which included the hippocampus, amygdale and the cortical regions that make up the parahippocampal gyri (reviewed in: Zola-Morgan and Squire, 1993;Corkin, 2002). For example, the well studied patient H.M was shown to retain the ability to learn new hand-eye co-ordination skills despite having no conscious memory of the training sessions, thus motor learning abilities had remained unaffected by the surgery (Corkin, 2002). Such findings were the first to suggest that the role of the hippocampus and related cortex in memory was much narrower than had been previously thought. It was finally suggested, following a series of hippocampal lesion studies in rats, that the hippocampus was specifically and solely involved in the conversion of short-term declarative memory into long-term declarative memory (Hirsh, 1974); this conversion process is now known as memory consolidation.

The initial studies of patients that had undergone surgical removal of the medial temporal lobes also provided strong evidence that the role of the hippocampus in memory is time-limited. While the patients were unable to form new long-term memories and showed a level of retrograde amnesia, their ability to recall remote autobiographical events remained comparable to that of control subjects (e.g. Scoville and Milner 1957;Squire et al., 1989). Moreover, the extent of retrograde amnesia in these and other amnesic patients was shown to be temporally graded, such that more recent memories were most affected (e.g. Zola-Morgan et al., 1986;Rempel-Clower et al., 1996). This led to the hypothesis that declarative memories become less and less dependent upon hippocampal function over time as a more permanent memory is gradually established elsewhere; most likely within the neocortex (Squire, 1992;Squire et al., 2001;Squire et al., 2004). Others studies however, have observed that a more extensive retrograde amnesia can also result from medial temporal lobe lesions and that memory loss can extend throughout a patient's lifetime (reviewed in Squire, 1992). Although it is possible that such cases simply reflect a more severe form of amnesia

resulting from more extensive brain pathology (Squire, 1992), it has also been proposed that these findings indicate that declarative memory retrieval and/or storage can remain depend upon hippocampal function no matter how old the memory (Sanders and Warrington, 1971;Nadel and Moscovitch, 2001;Cipolotti et al., 2001).

Nonetheless, results from brain lesion studies in animals have supported the proposal that memories may eventually become independent of hippocampal function. For instance, lesions to the hippocampus in rats and non-human primates have been found to result in temporally graded retrograde amnesia (e.g. Winocur, 1990;Zola-Morgan and Squire, 1990;Kim and Fanselow, 1992). Interestingly the length of retrograde amnesia was found to be different between animal species, being the shortest in rodents (1-3 weeks), intermediate in monkeys (2-12 weeks) and longest in humans (one to several years; reviewed in Squire, 1992). This suggests that the role of the hippocampus for memory in more recently evolved and complex vertebrates is more long-lived and that memory consolidation develops more slowly than in lower vertebrates (Squire, 1992); the longer times perhaps correlating with the more complex memory systems of higher vertebrates.

The brain locations to which memories are reorganised and the mechanisms that underlie this have not yet been identified, however a general hypothesis has emerged that after the initial hippocampal-dependent formation of a memory has taken place, the reactivation of hippocampal memory 'traces' may then drive cortical plasticity and thus transfer the memories to the cortex for more permanent storage. This popular theory has been referred to as 'synaptic re-entry reinforcement' (Wittenberg and Tsien, 2002).

One particular molecular genetic study has produced intriguing data that supports both the proposed temporary role of the hippocampus in memory storage and the possibility that memories are thereafter transferred to the cortex. It was found that heterozygous αCaMKII knock-out mice could form and retain two distinct types of hippocampus-dependent memory (spatial-reference and contextual) for 3 days after training but show clear amnesia compared to wild-type controls when tested 10-50 days later (Frankland et al., 2001). These animals exhibited normal LTP in the hippocampus but in the cortex LTP would decay rapidly back to baseline within an hour after induction. Thus, one

possible explanation of these findings is that the impaired cortical LTP meant that the acquired memory could not be successfully relocated to the cortex and hence resulted in the inability to retain the recently learnt information.

The hippocampal proper is also crucial for the encoding of spatial information, in particular of allocentric space (i.e. surrounding space that is independent of the subject's viewpoint). The current understanding about how the hippocampus may fulfil such a role has stemmed from the observation that specific hippocampal pyramidal neurones, termed 'place cells', respond selectively when the animal is positioned within a particular location (O'Keefe, 1979). The selectivity of these cells becomes more finely tuned and stable as an animal become more familiar with a particular environment. This correlates with the wealth of evidence indicating that hippocampal function is required in order for the animal to use its surrounding spatial cues in order to solve a spatial problem and to form spatial memories of its environment (e.g. Shaw and Aggleton, 1993;Cho et al., 1998;Burton et al., 2000;Deacon et al., 2001). It has even been proposed that the function of the rodent hippocampus may be dominated by its role in encoding spatial information (O'Keefe and Nadel, 1978). Note, animals also have the ability to navigate an environment using internal (egocentric) cues, a skill that is thought to be hippocampus-independent (Deacon et al., 2001).

It has also been suggested that elements of spatial memory may remain permanently dependent upon intact hippocampal function unlike other aspects of declarative memory that become hippocampal independent with time; this theory evolved from the finding that hippocampal lesions in animals can also result in temporally ungraded retrograde amnesia of previously learnt spatial memory (e.g. Mumby et al., 1999;Sutherland et al., 2001;Martin et al., 2005).

1.3 The Hippocampus: Anatomy & Circuitry

1.3.1 General Anatomy

The hippocampal formation (hippocampus) is an elongated structure located on the medial wall of the lateral ventricle whose longitudinal axis bends to form a C-shape. It consists of four distinct cortical regions: the hippocampal proper, the dentate gyrus, the

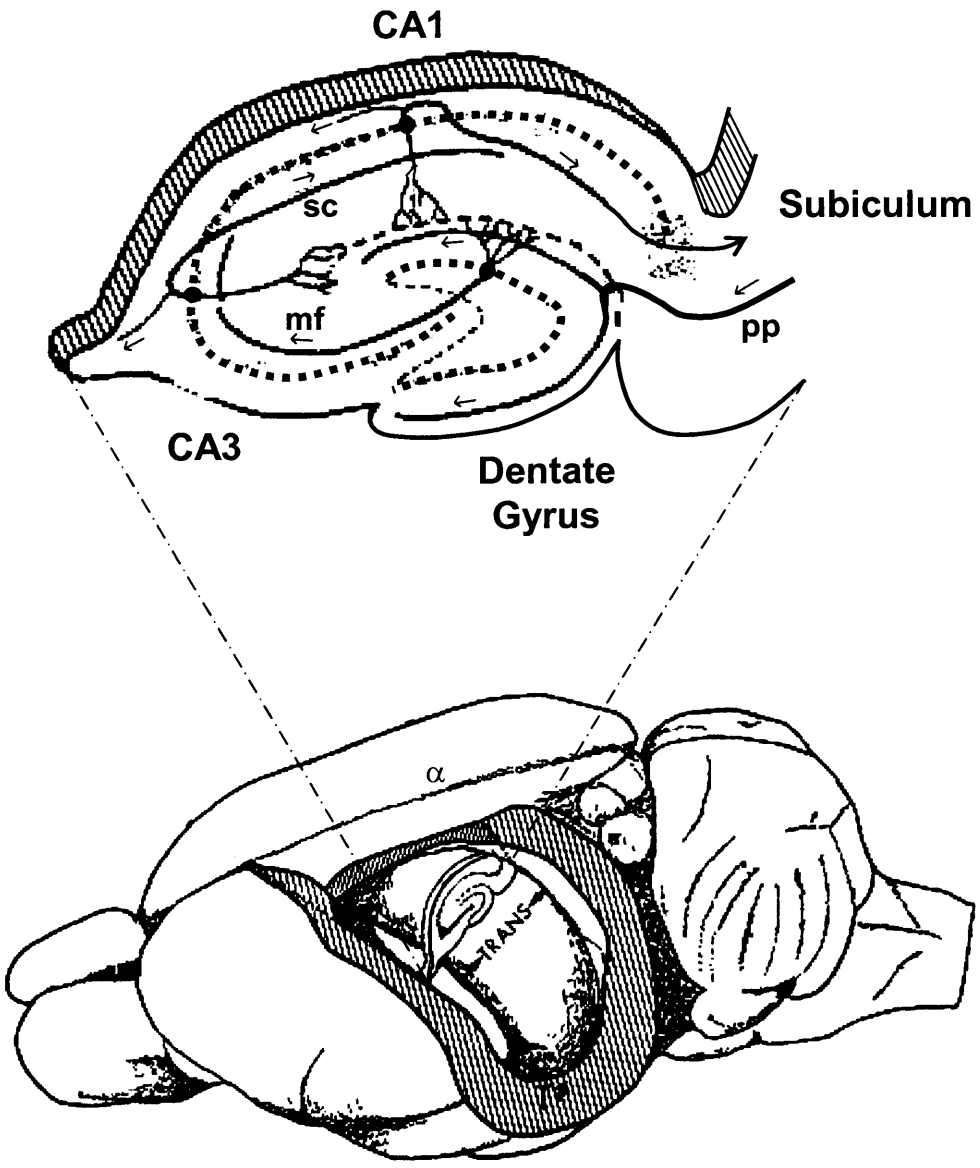
subicular complex and the entorhinal cortex. The subicular complex can be further divided into the subiculum, pre-subiculum and parasubiculum and, together with the entorhinal cortex, forms part of the parahippocampal gyrus and forms the convergence site for input from the various cortical areas that encode a declarative experience and mediates the organisation of cortical afferents into the hippocampus.

When the hippocampus is cut perpendicular to its longitudinal (septotemporal) axis to reveal a transverse section, all the constituent regions are clearly visible due to the layered arrangement of these structures (see figure 1.1).

The hippocampal proper is formed by an in-folding of the inferomedial part of the temporal lobe into the lateral ventricle. It is divided into three subfields, namely *cornu ammonis* 1 (CA1), CA2 and CA3. The hippocampal proper is also made up of distinct laminae: the *stratum oriens*, *stratum pyramidale*, *stratum radiatum* and *stratum lacunosum moleculare*. The principal cells of the hippocampal proper are excitatory pyramidal neurones whose cell bodies are arranged in a layer that forms the stratum *pyramidale*. The pyramidal neurones bear basal dendrites that arborise and form the *stratum oriens* and apical dendrites that descend into and radiate within the *stratum radiatum* and *stratum lacunosum moleculare*.

The dentate gyrus is also composed of several distinct layers: the molecular layer (*stratum moleculare*), the granule layer (*stratum granulosum*; populated by the cell bodies of the principal granule cells of the dentate gyrus) and the deep or polymorphic layer. The dendrites of the granule cells radiate within the molecular layer and the region that lies between the dentate gyrus and the hippocampal proper is called the hilus proper.

Figure 1.1 The Anatomy of the Hippocampal Formation



This figure shows a schematic of a transverse hippocampal brain slice and indicates the orientation of the hippocampal formation within the rodent brain. The location of each of the three principle cell layers of the hippocampal proper is represented by a coloured line: the pyramidal neurones of CA1 (red) and CA3 (green), and the granule neurones of the dentate gyrus (blue). The positioning of the intrinsic synaptic connections that make up the so-called trisynaptic pathway are also indicated: the perforant path (pp), the mossy fibres (mf) and the Schaffer collaterals (sc).

Adapted from Amaral and Witter, 1989.

1.3.2 Synaptic Connectivity within the Hippocampus

The hippocampal regions are synaptically connected by distinct and largely unidirectional projections. The pathways that are best characterised are those that make up a so-called trisynaptic loop. Briefly, this synaptic loop originates within the entorhinal cortex and projects to the principal cells of the dentate gyrus, through to hippocampal sub-region CA3, onto CA1 and then back to a distinct region within the entorhinal cortex. The synapses of this pathway are excitatory using glutamate as their transmitter and this pathway is considered to be the main information processing pathway of the hippocampus. A more detailed anatomical description, than that presented below, can be found in Amaral and Witter (1989).

The axons from layer II pyramidal neurones of the entorhinal cortex form the first synaptic projection of the trisynaptic pathway; the axons pass through the subicular complex and terminate upon the dendrites of the dentate granule cell. These axons make up the *indirect perforant path*. The granule cells then extend axons, via the hilus, to the CA3 subfield of the hippocampus where they make en passant synapses with the pyramidal cells of this region; these axons are referred to as *mossy fibres*. The presynaptic terminals of the mossy fibres form large varicosities that are thought to contain tens of release sites (Jonas et al., 1993). They form a unique and complex structure with the postsynaptic membrane that consists of intricate processes called *thorny excrescences*. CA3 neurones additionally receive a significant input direct from the layer II pyramidal neurones of the entorhinal cortex; these axons bypass the dentate gyrus (Amaral et al., 1990). As these layer II neurones also innervate the granule cells of the dentate gyrus, they therefore deliver qualitatively similar information to CA3 direct from the entorhinal cortex as is received by the granule cells and passed on again to CA3 via the mossy fibres (Treves, 2004). However, the properties of the perforant path and mossy fibres that impinge CA3 neurones are distinct (Ji and Staubli, 2002) and mossy fibres out-number the direct cortical projects by a factor of 50, highly suggestive of distinct functional roles (Treves, 2004). The axonal output of CA3 pyramidal neurones forms the major excitatory input to CA1 and, due to their highly collateralised nature, are known as *Schaffer collaterals*. This projection forms the third synapse of this well studied trisynaptic pathway. CA3 axons also form a dense network of associational synapses between CA3 pyramidal neurones and provide commissural connectively

between the two hippocampi via collaterals that exit the hippocampus through the fornix. It has been estimated that any one CA1 neurone may receive direct synaptic input from as many as 2% of the other CA3 pyramidal neurones from the two hippocampi (4% if only the ipsilateral CA3 neurones are counted Amaral et al., 1990). This high level of recurrent connectivity is thought to be a key feature that enables the hippocampus to bind information from multiple sites within the neocortex that together represent a whole memory (Squire, 1992). Recurrency is also responsible for rendering this region particularly unstable and vulnerable to epilepitiform activity – the spontaneous and hypersynchronous firing of a large number of excitatory neurones – thought to arise from a deficit of inhibitory synaptic activity (Bernard et al., 2000).

The Schaffer collateral to CA1 synapses are primarily located on the apical dendrites of CA1 neurones that lie within *stratum radiatum*, although the basal dendrites also receive some collateral input from CA3 (Ishizuka et al., 1990;Li et al., 1994) and some innervation from recurrent CA1 collaterals (e.g. Christian and Dudek, 1988;Thomson and Radpour, 1991). An additional input to CA1 is received by the apical dendrites that lie most distal to the CA1 somata. They receive their excitatory input exclusively via the *direct perforant path* that arises within layer III of the entorhinal cortex (Steward and Scoville, 1976). This pathway is thought to provide an important modulatory influence over CA1 output (Remondes and Schuman, 2002) that returns to distinct regions of the entorhinal cortex, layers IV and V, via the neurones of the subiculum (Amaral et al., 1991).

Within each of the hippocampal structures there are also inhibitory interneurones that use γ-aminobutyric acid (GABA) as their transmitter. In contrast to the rather uniform distribution of excitatory principal neurones within each sub-region, hippocampal interneurones display great variability, particularly in relation to their afferent and efferent connectivity, which is thought to permit this neuronal subtype to perform numerous tasks (Freund and Buzsáki, 1996). For example, interneurone activity is crucial for the generation and maintenance of excitatory network oscillations in the theta (4-12 Hz), gamma (40-100 Hz) and ultra-fast (200 Hz) frequency ranges; activities that are crucial for hippocampal function. The hippocampal interneurones have been classified into numerous families based their morphology, dendritic structure and axonal

targets (reviewed in Freund and Buzsaki, 1996).

The easily distinguishable regions of the hippocampus have lent it particularly suitable for physiological investigations. Moreover, the preservation of the synaptic connections that make up the trisynaptic pathway in the transverse hippocampal slice preparation (that can be kept alive for several hours *in vitro*) in addition to the ability of each of these distinct synapses to undergo bidirectional synaptic plasticity has rendered the hippocampal slice an ideal preparation in which to study the potential importance of plasticity for hippocampal function.

1.3.3 Excitatory synaptic transmission in the hippocampus

Excitatory synaptic transmission in the hippocampus is mediated by the actions of glutamate on both ionotropic and metabotropic receptors. The invasion of an action potential into a presynaptic terminal results in the stochastic fusion of a vesicle of glutamate with the presynaptic membrane and the release of its contents into the synaptic cleft. Glutamate is then able to diffuse within the cleft and bind to glutamatergic receptors located on the pre- and postsynaptic membranes. Glutamate may also escape the synapse and activate extra-synaptic receptors or, under some circumstances, even spill-over onto neighbouring synapses (Kullmann and Asztely, 1998). The majority of excitatory synapses in the brain, including those of the hippocampus, are formed onto small finger-like protrusions that arise from the postsynaptic dendrite (dendritic spines). Below the surface of the postsynaptic membrane within the spine head lies an electron-dense amorphous structure known as the postsynaptic density (PSD); the PSD is a specialization that in addition to providing the scaffold within which receptor proteins are clustered contains numerous signalling molecules that are ideally located therefore to detect and respond to incoming synaptic signals (Kennedy, 1997;Sheng, 2001;Yoshimura et al., 2004).

There are three distinct types of ionotropic receptors: NMDA receptors, AMPA receptors and kainate receptors; all are selective for cations and were initially distinguished based upon their distinct pharmacology (Watkins and Evans, 1981). The molecular and functional characteristics of each of these receptors and their many subtypes are now well defined (Kutsuwada et al., 1992;Seeburg, 1993;Dingledine et al.,

1999;Cull-Candy et al., 2001;Mayer and Armstrong, 2004); however, a detailed description of the distinct properties and functions of these receptor subtypes is beyond the scope of this thesis; a brief overview of their most important features are outlined below.

NMDA receptors are located on the postsynaptic membrane and they are characterised by their high permeability to Ca^{2+} (MacDermott et al., 1986) and their voltage-dependent channel block by extracellular Mg^{2+} (Nowak et al., 1984). This voltage-dependent block means that NMDA receptors are largely non-responsive to glutamate when the postsynaptic membrane is at or near to resting potentials and relief is only achieved when the postsynaptic membrane is sufficiently depolarised. This important property allows the NMDA receptor to act as a molecular coincidence detector of simultaneous pre- and post-synaptic activity as it is only activated if presynaptic glutamate release occurs at the same time as postsynaptic membrane depolarisation. The Ca^{2+} influx that occurs when NMDA receptors are activated underlies associative synaptic plasticity at various synapses within the hippocampus, including the perforant path and Schaffer collateral excitatory synapses of the trisynaptic circuit (Bliss and Collingridge, 1993). Indeed due to the above properties of this receptor subtype, their contribution to baseline synaptic transmission is minimal and their purpose is thought to be primarily for mediating changes in synaptic strength.

NMDA receptors are hetero-tetrameric proteins, composed from a variety of subunit proteins that have been identified and organised into 3 main subtypes: the NR1 subunit, that is ubiquitously expressed and present in all NMDA receptors; the NR2 subunit, of which there are four main types (NR2A-NR2D); and the NR3 subunits, that has two subtypes, A and B (Cull-Candy et al., 2001). Functional receptors are formed by the association of the NR1 subunit combined with at least one type of NR2. The different combinations of subunits determine the NMDA receptor properties such as single channel conductances, sensitivities to Mg^{2+}, relative affinities for glutamate and kinetics of the excitatory postsynaptic current (EPSC) waveform (Dingledine et al., 1999). In the hippocampus, as well as other cortical regions, the predominant NR2 subunits that accompany NR1 are NR2A and NR2B (Wenzel et al., 1997;Laurie et al., 1997). However, the NR2A subunits are only first detected around birth, and during postnatal

development a gradual replacement of the predominant NR2B with NR2A occurs (Kirson and Yaari, 1996;Stocca and Vicini, 1998;Ritter et al., 2002;Li et al., 2003) a switch that likely underlies the speeding of the decay phase of NMDA receptor responses (Carmignoto and Vicini, 1992;Crair and Malenka, 1995) and may have important implications for bidirectional synaptic plasticity through hippocampal development (Liu et al., 2004), namely the increasing difficulty in inducing LTD with low-frequency stimulation protocols (Errington et al., 1995;Kemp et al., 2000). Another important difference between the NR2A- and NR2B-containing receptors is with respects to their relative locations within the membrane. NR2A-containing receptors are primarily synaptic while the NR2B-containing receptors are thought to predominate at extra-synaptic sites (Stocca and Vicini, 1998;Tovar and Westbrook, 1999;Steigerwald et al., 2000).

AMPA receptors are voltage independent and the prime physiological function of these ion channels is to mediate fast glutamatergic synaptic transmission by allowing Na^+ to flux into and depolarise the postsynaptic cell (Seeburg, 1993). These receptors also exist as hetero-oligomeric proteins formed by the association of 4 subunits, GluR1-4. In the adult hippocampus, the receptor complexes that predominate are made of either GluR1 with GluR2 or GluR3 with GluR2 receptor subunits (Wenthold et al., 1996); in the developing hippocampus, GluR4 is also expressed (Zhu et al., 2000). The presence of the GluR2 subunit renders AMPA receptors impermeable to Ca^{2+} (Burnashev et al., 1992); thus, unless there is sufficient depolarising activity to relieve the NMDA receptor of its Mg^{2+} block, Ca^{2+} influx and the triggering of signalling cascades that can lead to activity-dependent plastic changes do not occur.

Important functional differences exist between the GluR1-GluR2 and GluR3-GluR2 AMPA receptors regarding their distinct synaptic trafficking mechanisms (e.g. Shi et al., 2001;Passafaro et al., 2001;Piccini and Malinow, 2002). Briefly, GluR2-GluR3 receptors undergo continual trafficking from non-synaptic to synaptic sites (Shi et al., 2001;Passafaro et al., 2001). This process is important for maintaining the basal levels of AMPA receptors within synapses. In contrast, the targeting of GluR1-GluR2 AMPA receptors to the postsynaptic membrane is an activity-dependent phenomenon and is a key mechanism underlying NMDA receptor-mediated increases in synaptic strength

(Shi et al., 1999). With time, these GluR1-GluR2 receptors are replaced with GluR2-GluR3 receptors; thus it is also thought that the continual trafficking GluR2-GluR3 receptors is also important for the long-term *maintenance* of activity-induced increases in synaptic strength and may be a mechanism by which synaptic strength is maintained in the face of protein turnover (Zhu et al., 2000;Shi et al., 2001). These subunit-specific trafficking processes are governed by the distinct proteins interactions made by the cytoplasmic tails of the different AMPA receptor subunits (reviewed in Bredt and Nicoll, 2003).

Kainate receptors are also found in hippocampal neurones, located most predominately within the CA3 hippocampal subfield and the dentate gyrus. They can form either homomeric or heteromeric tetramers (most likely heteromeric in their native form), assembled from the 5 different kainate receptor subtypes (GluR5-7, KA1 and KA2; Bettler and Mulle, 1995). Kainate receptors are located both pre- and post-synaptically and are most well known for their role in generating epilepitiform seizures in response to the application of kainic acid (Cherubini et al., 1983). Developing an understanding of the physiological roles of this subtype of glutamate receptor was initially restricted by the lack of antagonists that could select for kainate over AMPA receptors; however with the development of such compounds over the past decade some specific functions of are now slowly being unveiled (Lerma et al., 2001). In particular, presynaptic kainate receptors are now thought to play an important role in mediating a presynaptic form at LTP at the synapses made between mossy fibres and CA3 pyramidal neurones (Bortolotto et al., 2003).

Metabotropic glutamate receptors (mGluRs) belong to the group of G-protein coupled receptors. Eight different receptor subtypes have been identified to date (mGluR1-8) and are classified into three distinct groups according to their sequence homology, transduction mechanisms and pharmacology (Pin and Duvoisin, 1995). Group I mGluRs (mGluR1 and -5) are located to the postsynaptic membrane and agonism by glutamate leads to the activation of phospholipase C. On the other hand, both group II (mGluR2 and -3) and group III (mGluR4, -6, -7 and -8) mGluRs are located to presynaptic side of the cleft and are linked to the inhibition of cAMP-mediated signalling cascades. Thus although mGluRs are not themselves involved in fast excitatory synaptic transmission,

their activation by glutamate can exert diverse and profound effects upon synaptic and cellular physiology via their down-stream signalling cascades. For example, the excitability of hippocampal neurones can be directly modulated by the activation of Type I mGluRs and the phospholipase C-coupled regulation of various Ca^{2+} and K^{+} selective ion channels (Anwyl, 1999). The activation of mGluRs can also affect synaptic transmission, for example, through the inhibition of presynaptic Ca^{2+} currents that in turn results in the inhibition of transmitter release, as has been shown at the mGluR2/-3 expressing mossy fibre terminals (Kamiya et al., 1996). The mGluRs, like the other types of glutamate receptor, have also been shown to play important roles in various long-lasting forms of synaptic plasticity. Several distinct forms of mGluR-dependent LTD have been described in the hippocampus (Bortolotto et al., 1999;Nosyreva and Huber, 2005), and they may also play a modulatory role in LTP induction (Lynch, 2004).

1.4 Models of Hippocampal Processing for Memory Formation

Although there are many diverse views about the ways in which hippocampal circuits may support declarative memory, there are three common themes that compile a consensus about hippocampal functioning. These themes are based upon: 1) the elemental cognitive processes thought to be fulfilled by the hippocampus, 2) the characteristics of hippocampal circuitry and 3) the ability of excitatory connections within the hippocampus to modify their relative strengths in response to certain patterns of synaptic activity.

The key cognitive processes required for declarative memory formation thought to be mediated by the hippocampus are threefold (discussed in more detail in Eichenbaum, 2004). First is the ability to form associations between the various stimuli that make up a discrete memory (e.g. Gilbert and Kesner, 2002;Morris et al., 2003); for example, an episodic memory would require associations to be made between the relevant people that it involved, their actions, and the location where the event took place. The second is the ability to sequentially organise these representations so that the memory of an experience recalls the correct flow of events as they originally occurred (e.g. Holscher, 2003). The third is the ability of the hippocampal memory system to additionally organise the discrete factual elements of a memory into a relational network; this is

important because memories of facts or episodes do not exist in isolation, but instead they share many features with other memories that consist of similar information. A relational network has been proposed to offer two important properties (Eichenbaum, 2004): the ability to store factual elements of a memory in such a way that they are not bound solely to the episode in time from which they were acquired thus giving them additional "timeless semantic elements", and the ability to link common features of memories together, so that memories can be compared and contrasted and cross references made between memories rather than them being held as incomparable isolated events.

There are three specific properties of hippocampal circuitry that the various theoretical models have concluded could support these cognitive processes. The first is the high level of convergent afferent input that the hippocampus receives from the neocortex association areas (Amaral and Witter, 1989). This means that the granule cells and CA3 pyramidal cells receive high-level neural code about the experienced perceptions encoded by the cortex. The second important feature of hippocampal circuitry is the considerable level of recurrent connectivity within hippocampal sub-regions. As discussed above, this is particularly prominent in the CA3 subfield of the hippocampal proper, although CA1 (Christian and Dudek, 1988) as well as the dentate gyrus (Buckmaster and Schwartzkroin, 1994) have also been found to exhibit a level of recurrency among their populations of principal cells.

The third important property of hippocampal synapses, believed to enable these features of the circuitry to carry such cognitive functions and encode declarative memories, is the ability to undergo rapid changes in synaptic efficacy; a characteristic long recognised since the days of Cajal, Sherrington and then defined by Hebb (see below) as a process that could power neural processes and cognition. For instance, associative strengthening of multiple afferents to CA3 neurones could bind information from multiple sites within the neocortex that together represent numerous features of a memory, including information about attended stimuli and spatial cues (Squire, 1992). Furthermore, if only one cell representing only a fragment of an encoded memory within such a bound network were subsequently to fire, recurrent CA3 connections, strengthened by phenomena like 'LTP' (see below), would enable the entire neural

representation of a memory to be retrieved; a process known as 'pattern completion'. Similarly, computational models have emphasised that the plasticity of connections within recurrent networks of CA3 neurones ((Levy, 1996), as well as in the dentate gyrus (Lisman, 1999), could also support the temporal aspect of memory organisation through the strengthening of connections that always fire in sequence. Thus, if an input that is linked to just one temporal element of a memory is triggered during recall, the network would be able to place that representation within the correct sequence of recalled events. These same recurrent network properties able to encode the temporal aspects of memory may also support a mechanism that is able to link together similar features of different memories; thus forming memory 'relational networks'. Such a network would enable the hippocampus to form associations among related memories that could, in turn, bring added meaning to other, more ambiguous, contexts (Eichenbaum, 2004). This element of cognition would enable animals to draw parallels between similar but distinct contexts, enhancing their chances of survival.

The role of the CA1 subfield as a stage of hippocampal processing is less clear. Unlike the CA3 pyramidal neurones, those in CA1 do not receive mossy fibre inputs, nor do they give rise to such an extensive network of recurrent collaterals. It has been suggested that the CA1 output to the subiculum, the principal target of CA1 cell axons (Amaral et al., 1991) which also receives and integrates incoming hippocampal information from various cortical regions, may compare predictions of the hippocampal network regarding sequenced information with incoming sequences as the information arrives (Eichenbaum, 2004). However, the prime function of the CA1 output is considered to be one that decodes the signals arising from CA3 back to the cortex, an interface that, in context of present theories, is important for translating the temporary hippocampal memory into a more permanent cortical store. As the subiculum and entorhinal cortex transmit the information between CA1 and the cortex, plasticity at the CA1 inputs to these regions, in particular the subiculum (e.g. O'Mara et al., 2000) is also likely to play an important role in memory consolidation (e.g. Rolls, 1996).

Studies have also suggested that CA1 plays a specific role in establishing memory representation of specific spatial cues in a way that is independent of dentate gyrus and CA3 processing (Jarrard, 1995). This theory was based on the observation that selective

ablation of either dentate granule cells (McNaughton et al., 1989) or CA3 pyramidal neurones (Brun et al., 2002) does not abolish the specificity of CA1 place cells.

1.5 Long-Lasting Changes in Synaptic Efficacy: the Substrate for Learning & Memory in the Hippocampus

1.5.1 The Discovery of Long-Term Potentiation (LTP)

The search for cellular and molecular substrates of hippocampal-dependent learning has made much progress in the recent decades. Huge propulsion for research into this field was fueled by the discovery that brief high-frequency stimulation of the rabbit perforant pathway caused a rapid and sustained increase in the synaptic efficacy of granule cells in the dentate gyrus (Bliss and Lomo, 1973;Bliss and Gardner-Medwin, 1973). These were the first findings to establish a biological analogue of the earlier postulate, formulated with much fore-sight by Donald Hebb (1949), that stated:

"When an axon of cell A... excite(s) cell B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells so that A's efficiency as one of the cells firing B is increased."

LTP has since been demonstrated at all excitatory pathways within the hippocampus, as well as in many other brain regions and remains to be regarded as the most likely of cellular mechanisms to underlie learning and the formation of memories in the brain. It is also widely agreed that such a phenomenon could play a fundamental role in the refinement of neural circuitry during development (Goodman and Shatz, 1993;Katz and Shatz, 1996;Constantine-Paton and Cline, 1998;Huh et al., 2000;Katz and Crowley, 2002).

1.5.2 Essential Properties of LTP

There are several basic properties of LTP that are considered to make it a suitable neural substrate for learning and memory; it exhibits: input specificity, associativity, cooperativity and the ability to persist for long-periods of time (Bliss and Collingridge, 1993;Malenka and Nicoll, 1999). Input specificity means that only the inputs on a neurone that receive the appropriate synaptic stimulation will exhibit LTP, and other

inputs on the same cell are unlikely to be affected; note however, that it has also been suggested that this 'homosynaptic' rule may only apply to synapses that are not in close proximity to the stimulated pathways (Engert and Bonhoeffer, 1997). Nonetheless, such specificity would greatly increase the theoretical computational power of individual neurones as well as the storage capacity for memory. Associativity refers to the fact that if one set of synapses onto a cell is strongly activated this can facilitate LTP at an independent set of converging synapses; associative LTP at the converging synapses may occur even if their input is weak so long as both sets of inputs are active at the same time. It is thought that this property is relevant for the associative nature of learning, like that implicit to classical conditioning; associativity is also a key aspect of the theoretical 'Hebbian' synapse. Cooperativity, is similar to associativity in that it involves the fact that a stimulation threshold exists for LTP induction, however, LTP is cooperative because it can be induced by the strong stimulation of a single pathway, or by the weak stimulation of many. This is an important characteristic for theoretical models of learning in the brain. Finally, it is generally considered that if the neural substrate of memory lies in the modification of synaptic strength, then such changes must be able to persist for the duration of that memory. LTP in the hippocampus has repeatedly been shown capable of lasting hours *in vitro*, months and even up to a year *in vivo* (Abraham et al., 2002). The magnitude of LTP is well documented however to decay to an asymptotic level over these longer periods, although the decremented levels can be topped back up to the original degree of potentiation with further stimulation (e.g. de Jonge and Racine, 1985). Nevertheless, it still remains a matter of great debate whether such prolonged persistence in rodents is relevant for the functioning of hippocampus (Martin and Morris, 2002), particularly as its role in memory may only be temporary (Squire, 1992).

1.5.3 Long-Term Depression

In addition to the strengthening of synaptic connections, the activity-dependent and weakening of synaptic efficacies has also been characterised at each of the excitatory synapses with the hippocampal circuitry (Malenka and Bear, 2004). LTD has now been characterised at many synapse types within a number of brain regions, and like LTP it is considered to be a fundamental process contributing to various brain functions (Kemp and Bashir, 2001). Unlike LTP which was first identified and is still typically induced

by stimulating afferent inputs with high-frequency stimulation protocols (usually short bursts of electrical stimuli at 100 Hz or higher), the induction of homosynaptic long-term depression (LTD) was discovered by applying low-frequency stimulation to afferent pathways (typically 1 Hz for 900 seconds; Dudek and Bear, 1992;Dudek and Bear, 1993). LTD also exhibits each of the three criteria considered essential for a neural correlate of memory (associativity, specificity and persistence) and may also therefore provide a cellular basis for learning and memory in the brain (Braunewell and Manahan-Vaughan, 2001). Indeed, LTD is widely considered as a critical process for cerebellar-dependent motor learning, resulting in improved smoothness and accuracy of movements (Boyden et al., 2004). LTD and has also been associated with specific aspects of hippocampal-dependent processing, including acquisition of object-place associations (Kemp and Manahan-Vaughan, 2004).

Although the fundamental need for a brain process that results in the weakening of synaptic strengths was always widely accepted, the role of LTD as a mechanism for learning and memory has received far less support than LTP. The suspicion has been based on two main points. The first is that LTD was found to be notoriously difficult to induce (particularly *in vivo*) therefore its legitimacy as a physiological phenomenon was doubted, and second, the initial induction protocol identified required up to 15 minutes to result in stable depression – an unlikely characteristic of a processes underlying rapid learning and memory formation. As research has progressed into this field, the justification behind the first doubt has now been discredited, as LTD induction has been repeatedly demonstrated in adult as well as young animals, *in vitro* and importantly *in vivo*. What is also clear now is that more than one form of synaptic depression can exist at a particular synapse, that the ability to induce LTD can be extremely sensitive to experimental conditions and that the induction protocols required to recruit the appropriate molecular machinery to induce LTD changes with age (Kemp et al., 2000;Nosyreva and Huber, 2005).

In response to the second doubt, specific induction protocols and conditions have now been identified that are capable of inducing rapid homosynaptic LTD, which may, therefore, resemble more physiological forms of depression. For instance, Huerta and Lisman were able to induce LTD by delivering 4 electrical pulses at 100 Hz applied

during the negative peak of a carbachol-induced theta frequency oscillation in a hippocampal slice (Huerta and Lisman, 1995). This study also found that if the stimuli were applied during the peak of an oscillation LTP was produced, supporting the idea that such induction protocols may resemble more physiological-like forms of bidirectional plasticity. Hippocampal theta-rhythm activities have indeed been proposed to be important for the hippocampal processing of new information (Buzsaki, 1989), and the occurrence of *in vivo* hippocampal theta rhythms have been shown to increase when animals are exposed to novel learning environments (Xu et al., 1998). Furthermore, LTP induced in the CA1 region *in vivo* is reversed when the rats are then allowed to explore a novel environment but not when they enter a familiar one (Xu et al., 1998), suggesting that mechanisms that decrease synaptic efficacy (or, at least, reverse non-meaningful/unconsolidated potentiated synapses) are activity recruited during learning situations. Moreover, the exploration of novel environments has been shown to facilitate the induction of LTD with low-frequency stimulation in CA1 *in vivo* (Manahan-Vaughan and Braunewell, 1999;Kemp and Manahan-Vaughan, 2004). Together these findings support the possibility that LTD could represent a mechanism underlying certain forms of learning and memory.

More recently, alternative stimulation protocols have been identified that can support the rapid induction of LTD as well as LTP; thus again rebuking the concern that LTD could not underlie a rapid learning mechanism. A paradigm, known as spike-timing-dependent plasticity (STDP), has been established where depending on the precise timing (on the scale of milliseconds) and temporal order of pre- and post-synaptic activity, either LTD or LTP can be induced. LTP occurs when a single presynaptic action potential precedes a postsynaptic one, while the reverse order of activity produces LTD (e.g. Magee and Johnston, 1997;Schuett et al., 2001;Froemke and Dan, 2002;Wang et al., 2005).

It is now generally accepted, that both LTD- and LTP-like phenomena are likely to exist physiologically and that they may each be required for distinct aspects of hippocampal function that together underlie the processes required for learning and memory formation. Indeed, other forms of activity-dependent synaptic plasticity are also likely to be intimately involved in hippocampal functions related to learning. Such forms

include: *heterosynaptic LTD*, a process that is triggered by postsynaptic depolarisation at inputs other that the resultant depressed ones (Lynch et al., 1977); *de-potentiation*, the activity-dependent (and time-limited) reversal of LTP – a process that recruits molecular pathways distinct from those that underlie LTD expression at naïve synapses (Barrionuevo et al., 1980;Staubli and Lynch, 1990); forms of *short-term plasticity*, like short-term facilitation and depression (Zucker and Regehr, 2002); *metaplasticity*, the modification of activation thresholds for LTP and LTD induction (along a sliding scale) that depends upon the previous history of neuronal activity (Abraham and Bear, 1996); and forms of *homeostatic plasticity* (or '*homeoplasticty*'), a form of plasticity that strives to maintain the total levels of excitatory input to a single cell within an optimal range and is able to cause global changes in synaptic strengths throughout an entire neurone (Turrigiano and Nelson, 2000).

1.6 Evidence that Synaptic Weight Changes are required for Hippocampal-Dependent Learning & Memory Formation

To uphold the hypothesis that synaptic plasticity is the physiological correlate of learning and the formation of memories, a set of criteria has been devised which must first be satisfied (Martin et al., 2000;Martin and Morris, 2002).

1) *Detectability*: The first criterion is that after an animal has acquired a new memory, changes in synaptic weights should be detectable in the brain and that interventions that reverse the identified changes should erase the memory.

2) *Intervention*: The second criterion is that interfering with the mechanisms that enable synapses to undergo plastic change should impair the ability of an animal to acquire new memories. This approach has received the most experimental attention as it has come hand-in-hand with experiments that seek to identify the cellular pathways and molecules that underlie the expression of phenomena like LTD and LTP.

3) Mimicry: The final criterion, and one that is yet to be exemplified, is that it should be possible to 'implant' a memory of an association or of an event into an animal's brain by experimentally inducing an appropriate pattern of synaptic weight changes.

A huge variety of experimental approaches have been used over the past decades to investigate whether these criteria can be met (reviewed inMartin and Morris, 2002).

1.6.1 Detecting Synaptic Weight Changes Subsequent to a Learning Experience

One line of investigate that has been taken to investigate the first criterion was to see if the acquisition of new memories in a rat (as it explored a novel environment) correlated with changes in the efficacy of field excitatory postsynaptic potentials (EPSPs) recorded in the dentate gyrus (Moser, 1995). Increases in synaptic efficacy were identified suggesting that potentiation of synaptic responses *is* involved in dentate function and the acquisition of new memories; however, the interpretation of these data has been highly controversial and their validity viewed with much skepticism. This is due to the finding that physical activity in the behaving animals led to increases in brain temperature that can also cause increases in dentate field potentials, rendering changes that may be associated to true memory-related processes difficult to resolve (Pettit et al., 1994;Andersen and Moser, 1995).

A slightly different line of approach for detecting evidence of learning-induced plasticity has, however, been more successful. For example, studies have found that the evoked field potentials in the dentate gyrus of hippocampal slices taken from rats reared in enriched environments are greater than the field potentials recorded in hippocampal slices from rats housed in standard isolated conditions (Green and Greenough, 1986;Foster et al., 1996). As mentioned earlier, enriched environments involve larger cages filled with a variety of novel objects and materials that provide the opportunity for animals to engage hippocampal-dependent processes like the formation of spatial and declarative memories. As synaptic potentials were assessed *in vitro*, the responses could not have been affected by temperature related artifacts as observed in the dentate gyrus *in vivo*. It was also found that the magnitude of LTP that could be induced in these preparations was substantially reduced. This partial occlusion of LTP suggests that the increased baseline field EPSPs could have been caused by enrichment-mediated processes that involve the same cellular processes that are required for LTP expression *in vitro* (Foster et al. 1996). Importantly, the enrichment-mediated enhancement of field EPSPs and reduced magnitudes of LTP were blocked in rats that received a chronic

intracerebroventricular perfusion of the NMDA receptor antagonist AP5 during enrichment (Foster et al., 2000), a finding that is consistent with the proposal that the observed changes in enriched rats were mediated through NMDA receptor-dependent processes. However, alternative enrichment-mediated processes could also have contributed to the increased dentate field potentials. For example, learning, physical exercise and environmental enrichment have been shown to enhance neurogenesis in the dentate gyrus as well as the survival of the newly born neurones (Nilsson et al., 1999;Gould et al., 1999;Van Praag et al., 1999b), thus increased synaptogenesis could also explain the increased dentate field potentials in enriched rats. Indeed, learning a spatial task has been found to result in greater spine densities within the dentate gyrus of rats (O'Malley et al., 2000). Note, LTP in the organotypic slice cultures has also been associated with the appearance of new dendritic spines within the stimulation region of dendrite (e.g. Engert and Bonhoeffer, 1999), thus synaptogenesis between existing neurones might also contribute towards the enhanced synaptic potentials observed following environmental enrichment.

Changes in synaptic efficacies have also been linked more directly to learning and the acquisition of memories. Training rabbits in an eye-blink conditioning task has been shown to result in enhanced CA1 responses evoked in brain slices taken shortly after training (Power et al., 1997). Similarly, conditioning rats to associate a particular spatial context to a foot shock (a fear learning paradigm that requires the amygdala as well as the hippocampus; Phillips and LeDoux, 1992) has also been shown to increase CA1 field responses in hippocampal slices. These increases could be detected in slices taken from rats that were sacrificed up to 7 days after conditioning (Sacchetti et al., 2001). Fear-conditioning also inhibited the ability to induce further potentiation using LTP-inducing stimuli, although this partial occlusion of LTP was only observed in slices taken within 24 hours after training (Sacchetti et al., 2002). Further changes must therefore take place *in vivo* that re-establish the potential to induce LTP at the later time-points whilst maintaining the enhanced field potentials that could be seen for as long as 7 days. This could again reflect a learning-mediated synaptogenesis in the hippocampus which could reestablish the LTP capacity in CA1 to its initial magnitude. Indeed, the density of synapses has been found to increase within *stratum radiatum* of CA1 following environmental enrichment in mice (Rampon et al., 2000b, but see Moser

et al., 1997) and to correlate with the late-phase of LTP induced *in vitro* (Chang and Greenough, 1984;Chang et al., 1991;Bozdagi et al., 2000).

However, the idea that any measured increases in evoked field potentials that follow either enrichment or the acquisition of memory with formal training could relate to actual memory has also been met with much cynicism. This is largely based upon the theoretical predication that only a few synapses in the hippocampal network should undergo weight changes in order to enable the hippocampus to yield a high enough storage capacity for declarative memory; i.e. the necessity of a sparse code (Willshaw and Dayan, 1990) – hence the phrase, "if you can measure it, it's not memory"! An alternative interpretation might be that the detected changes in synaptic weight may reflect more general changes that were related to information processing rather than memory itself (Martin and Morris, 2002). Thus, although the likelihood of detecting changes that correlate with the neural substrate of memory itself maybe small, other changes may be detected that accompany the processes required for the hippocampal function. Indeed recent evidence has suggested that the proportion of hippocampal CA1 neurones that are activated during an exposure to a novel environment may be as high as 35-40% (Guzowski et al., 1999;Vazdarjanova and Guzowski, 2004), although the proportion and distribution of synapses that may have undergone long-lasting changes in synaptic efficacy on these activated neurones remains unknown.

Experiments that aim to reverse the specific weight changes, induced by a prior learning experience in order to see whether it alters an animal's memory of that experience (i.e. the second half of the first criterion), would help determine whether the measured synaptic weight changes that accompany memory acquisition could underlie memory or not. The difficulty again lies in the ability to detect the specific synapses that directly relate to the acquired memory and then in the ability to reverse those changes selectively. Although this hasn't been tested to date so precisely, more general interventions have, however, been applied to gather evidence that alludes towards this criterion. One study showed that after rats had successfully learnt the location of a

submerged platform in the Morris water maze paradigm*, applying high-frequency stimulation across the tract of perforant path fibres *in vivo* resulted in the inability of the animals to relocate the platform on a subsequent probe trial (Brun et al., 2001). This apparent memory loss by the artificial induction of LTP, and the presumed scrambling of the neural code, was prevented if the tetanisation occurred in the presence of the NMDA receptor antagonist CPP (Brun et al., 2001).

Evidence that learning results in synaptic weight changes has also been gathered from various other brain regions including the amygdala (e.g. Rogan et al., 1997;Collins and Pare, 2000) and various regions of the neocortex (e.g. Roman et al., 1993;Rioult-Pedotti et al., 2000;Lebel et al., 2001;Mouly et al., 2001; reviewed in depth in Martin and Morris, 2002). In particular, a large field of study has focused on the role of plasticity in the establishment of sensory maps and receptive field properties in the somatosensory cortex. Such studies include those that investigate the critical periods during development when sensory experience plays a crucial role in shaping the anatomical and functional properties of synaptic connectivity in developing cortex (Fox, 2002;Calford, 2002;Jones et al., 2002;Katz and Crowley, 2002). Altogether, these findings strengthen the proposal that the plasticity of synaptic connections occurs in the brain and is a phenomenon that underpins numerous brain functions including brain development and the various forms of learning, as well as declarative memory formation.

1.6.2 Plasticity Impairments and Memory

Studies that investigate whether synaptic plasticity is the cellular correlate for memory have largely focused on the second criterion; i.e. applying interventions that inhibit the expression of long-lived synaptic plasticity in order to investigate whether the impairments correlate with learning and memory impairments. As with the other assessment criteria, a wealth of such data is now available regarding the various

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* The Morris water maze is the most commonly used test of spatial reference memory in rodents and requires the animals to learn, over multiple trials, to locate the position of a hidden platform using distal spatial cues to navigate (Morris et al., 1982;Morris, 2003)

different memory systems of the brain, however for this thesis I will only touch upon those concerned with hippocampal-dependent learning and memory.

Pharmacological and genetic techniques have presented numerous tools with which an experimenter can modify the function of a particular molecule and then correlate any changes in the properties of experimental synaptic plasticity (compared either to the same animal before treatment or to untreated controls) with changes in the animals' performances in behavioural tests of memory. This approach has been highly successful in yielding a wealth of evidence correlating parallel deficits in hippocampal LTP with learning and memory. In particular, the selective disruption of plasticity at specific afferent pathways or of specific forms of plasticity (defined for example by their induction requirements and downstream signalling molecules) has helped to reveal information about different cognitive forms and temporal stages of memory formation (e.g. Martin and Morris, 2002).

For example, the *in vivo* administration of competitive NMDA receptor antagonists into rats during training (via systemic, intracerebroventricular or intrahippocampal drug infusion systems) has been shown to severely impair their ability to learn in a variety of different hippocampal-dependent learning paradigms (e.g. Morris et al., 1986;Morris, 1989;Tonkiss and Rawlins, 1991;Fanselow et al., 1994;Steele and Morris, 1999). However, in addition to blocking the induction of certain forms of synaptic plasticity, NMDA receptor blockers were recognised to have a number of other physiological and behavioural effects which therefore confounded the interpretation of these data (Walker and Gold, 1994;Cain et al., 1996;Saucier et al., 1996). Nevertheless, additional support for the role of NMDA receptor-mediated synaptic plasticity in learning came from the development of genetic techniques that enabled the targeted disruption of NMDA receptor-mediated synaptic transmission in more spatially restricted ways. For example, the selective knock-out of the NR1 NMDA receptor subunit (which is required for the formation of functional receptors) in the CA1 hippocampal subfield in mice resulted in the loss of LTP in this region and impaired their performance in tests of spatial memory (Tsien et al., 1996). Another study selectively knocked out NMDA receptors in the CA3 region, which selectively impaired LTP at the CA3 recurrent commissural/associational synapses (Nakazawa et al., 2002). These mice were able to form long-term spatial

reference memories in the standard Morris water maze test but they were severely impaired when some of the spatial cues were removed. This finding was consistent with the hypothesis that CA3 recurrency performs a 'pattern completion' function, i.e. the ability to retrieve a complete memory following the presentation of a partial set of cues (Nakazawa et al., 2002). Further investigations found that they were also selectively impaired in short-term memory tests of novel one-time experiences (Nakazawa et al., 2003); indicating that the rapid acquisition and storage of information from the single exposure to spatial cues also replies upon intact plasticity at these inputs. This form of one-time-experience learning in rodents is also referred to as 'working memory' and may be akin with human episodic memory (the rapid and perpetual acquisition of memories that record each unique event in time and retains the ability to distinguish each event from similar past events; Tulving and Markowitsch, 1998). Such investigations were not only crucial for demonstrating the critical role of NMDA receptors in synaptic plasticity and learning but also for dissecting out the contribution of synaptic pathways for distinct aspects and types of learning.

A large number of studies have also targeted the molecules downstream of synaptic transmission that have been identified from *in vitro* studies to be involved in the expression or maintenance of either LTP or LTD in order to correlate the potential importance of such pathways for *in vivo* plasticity, learning and memory. For example, through the use of gene technology various studies have been able to correlate the aberrant synaptic plasticity in mice with learning and memory deficits that arise from intervening with the function of key enzymes, including: the protein kinases CaMKII and PKA (cAMP-dependent protein kinase), which play important roles in LTP signalling cascades; the protein phosphatase 2B (PP2B; also known as calcineurin) that is required for LTD expression; and cAMP response element-binding protein (CREB), a nuclear transcription factor, the action of which is required for the late-phases of plasticity and is specifically correlated with the long-term persistence of memory (reviews include: Silva et al., 1998;Silva, 2003;Mansuy, 2003;Elgersma et al., 2004).

In fact, the first ever use of gene knock-out technology in biology was specifically used to probe the relationship between LTP and memory. The gene targeted was that for the α-subunit of CaMKII which resulted in the complete loss of protein expression and the

knock-out animals showed significant impairments in both CA1 LTP and in hippocampal-dependent spatial-reference memory (Silva et al., 1992a;Silva et al., 1992b). This was a highly significant finding of the time, adding considerable strength to the hypothesis that the same mechanisms that underlie LTP were required for learning and memory processes in the behaving animal. A following study that blocked the ability of this kinase to undergo autophosphorylation (Giese et al., 1998), a property that theoretical models of synaptic plasticity had proposed was fundamental for its role in synaptic plasticity (Lisman, 1989;Lisman, 1994), was then also able to link this specific property of the kinase to the resulting LTP and memory impairments exhibited by these mice.

Although the majority of studies have focused on the association of increases in synaptic strength with memory, neural network models have suggested that reductions in synaptic efficacy would also be important for learning (Willshaw and Dayan, 1990). In accordance with this prediction, evidence from a specific PP2B-knock-out mouse has also implicated a specific role of LTD-like phenomenon in hippocampal memory (Zeng et al., 2001). In the study by Zeng et al. (2001), ablation of PP2B activity was achieved by knocking-out the gene for its regulatory subunit that is required for activity of either of the two PP2B catalytic subunit isoforms. Adult mice possessed a selective impairment in LTD induction but retained normal LTP and depotentiation (phosphatase-dependent reversal of LTP). The performance of the mutants in tests hippocampal-dependent spatial reference memory (including the Morris water maze and contextual-fear conditioning) were unaffected and correlated with intact LTP; however, the mutant mice were severely impaired in a spatial-memory task that required the learning of a series of successive novel locations (the delayed matching-to-place task; see Chen et al., 2000a) and in the working memory version of an 8-arm radial maze task. These findings suggested that LTD may specifically play an important role in tasks requiring flexible learning of new information, together with the rapid extinction (i.e. unlearning) of information that is no longer required (Martin and Morris, 2002). It would be interesting to see if and how the properties of bidirectional synaptic plasticity are affected at CA3 synapses in these mice since, as discussed above, NMDA receptor function in CA3 neurones has been identified as being particularly important for

working/episodic-like memory (Nakazawa et al., 2003).

The underlying mechanisms of synaptic depression are also thought to provide a negative constraint over the mechanisms that lead to synaptic potentiation and hence the memory processes that require enhanced synaptic strengths (reviewed in Mansuy, 2003). Thus, the overcoming or the active removal of this constraint may itself represent an important feature in the control of long-term memory formation. For example, it may provide a mechanism by which the brain is able to differentiate between relevant experiences that are to be processed into long-term memory and more insignificant experiences that will not persist (Silva and Josselyn, 2002). In accordance with this, the partial inhibition of PP2B activity in transgenic mice with one of the catalytic subunits knocked-out was found to enhance both LTP and the performance of mice in tests of spatial and object recognition memory (Malleret et al., 2001). LTD in these mice remained unimpaired. Another study generated mice where the expression of a constitutively active inhibitor of a different protein phosphatase (PP1 – which is also required for LTD induction, see below) could be switched on and off in a controlled manner (Genoux et al., 2002). Expression of this transgene, and thus reduced PP1 activity, enhanced memory acquisition and the persistence of hippocampal-dependent memories compared to wild-types and to mutants where the transgene remained inactive.

Note, although numerous pharmacological and genetic studies have now demonstrated a correlation between enhanced LTP and learning and memory abilities, this is not a required criterion that needs to be met in order to satisfy the hypothesis that synaptic plasticity is the physiological correlate of learning and memory formation (Martin and Morris, 2002). Another important point to mention is that even though learning and memory impairments may occur after interfering with the function of a specific molecule that is known to be required for the induction or expression of experimental plasticity (like LTP or LTD) this does not necessarily mean that phenomena similar to either LTD or LTP also occur *in vivo*; for example, the specific protein may be involved in forms of plasticity that are quite distinct to these experimental paradigms, or other functions of the protein not related to synaptic plasticity may be affected that are also necessary for memory. Such findings can be used however, to suggest that the processes

underlying experimental and endogenous plasticity may be closely linked. On the other hand, the lack of a behavioural phenotype may not necessarily mean that the molecule of question is not normally involved in the mechanisms underlying learning and memory, but it could be that other molecules can compensate for the loss or that its action is not critical for endogenous plasticity.

In addition, even though there has been a wealth of evidence linking the loss of experimental forms of plasticity with cognitive impairments, there have also been several curious cases where deficits in LTP have not been accompanied by altered learning abilities (Huang et al., 1995;Nosten-Bertrand et al., 1996;Okabe et al., 1998;Zamanillo et al., 1999). For example, mice with the GluR1 AMPA receptor subunit knocked-out show a complete lack of LTP in the CA1 region in acute brain slices yet are able to learn a spatial-reference memory task in the Morris water maze as fast as controls (Zamanillo et al., 1999). The animals in this study underwent an extensive training, thus one possibility is that they were able to develop CA1 independent-learning strategies over the multiple training sessions which could have attenuated the requirement for plasticity in this task (Saucier and Cain, 1995). Another study selectively ablated specific PKA isoforms using gene targeting techniques and observed a selective deficit in mossy fibre to CA3 LTP and although other studies have suggested that plasticity at this pathway may be required for spatial learning (e.g. Sandin et al., 1998;Lassalle et al., 2000;Ramirez-Amaya et al., 2001;Daumas et al., 2004) these animals showed no deficits in tests of hippocampal-dependent spatial reference and contextual memory (Huang et al., 1995). An explanation could be that in spite of the apparent loss of experimentally induced plasticity, endogenous forms of synaptic plasticity are still intact. For example, in the mutant mouse lacking cell adhesion molecule Thy-1, LTP assessed in anesthetised animals *in vivo* was strongly inhibited in the dentate gyrus (albeit in tact in CA1) yet these animals showed normal spatial learning as assessed in the Morris water maze (Nosten-Bertrand et al., 1996). However, in a later study when synaptic plasticity was assessed in freely moving Thy-1 mice, much greater levels of LTP could be induced (Errington et al., 1997).

Martin and Morris (2002) have outlined several reasons why the failure to be able to induce synaptic plasticity may not necessarily reflect the loss of learning-induced

plasticity *in vivo*: 1) since our current understanding of neuronal firing patterns in behaving animals is limited, the conditions and activity patterns *in vivo* may still have the capacity to induce experience-driven plasticity, whereas the stimulation protocols used experimentally may no longer be sufficient to provoke synaptic plasticity in the subject preparation (i.e. one that has undergone drug or genetic manipulation); 2) it is possible that a different form of plasticity is required at the synapse in question for the type of learning that is being assessed; e.g. synaptic depression rather than potentiation; 3) testing plasticity *in vitro* or in anaesthetised animals *in vivo* may produce different results to those assessed in behaving animals, as was the case for the Thy-1 mutant (Errington et al., 1997); 4) plasticity in untested pathways may be critical for the specific form of learning and memory that was assessed. Despite these problems, the use of the growing number and increased specificity of interventions that can selectively modify the action of molecules in spatially and now also temporally restricted ways (Garcia and Mills, 2002), still provides a highly valuable method with which to develop our understanding of the specific types of plasticity and synaptic pathways that are involved in the distinct types and stages of learning and memory formation.

Another strategy that has been used to study whether activity-driven increases in synaptic weights in the hippocampus are necessary for learning has been to study the behavioural consequences of driving synaptic potentiation to its apparent maximum, i.e. of saturating LTP. If further LTP-like experience-driven increases in synaptic weights are necessary for memory encoding in the hippocampus then the saturation of LTP in the hippocampus should impair learning. Indeed it has been shown that saturating synaptic efficacy at perforant path inputs before exposing the animals to a learning task is able to interfere with hippocampus-dependent learning and the acquisition of spatial memories (McNaughton et al., 1986;Castro et al., 1989;Barnes et al., 1994;Moser et al., 1998). Some studies however, failed to observe a deficit in learning after tetanisation of the perforant path (e.g. Sutherland et al., 1993;Jeffery and Morris, 1993), but these results have since been attributed to the stimulation of too few of the afferent fibres (Moser and Moser, 1999).

1.6.3 Mimicry: Experimental Memory Induction

If a new memory can be generated artificially through the experimental alteration of

synaptic weights without the usual requirement for sensory experience then this would be the ultimate demonstration that synaptic plasticity is the true cellular correlate of learning and memory in the brain. However, this is obviously easier said than done and a successful mimicry experiment has not been achieved to date.

Considering the complexity of declarative memory systems this seems to be a challenge that may be impossible to conquer, however, it may be possible if applied to simpler forms of memory and in less complex memory and nervous systems of lower organisms. Various studies have made steps towards mimicry by studying forms of associative conditioning. Conditioning exploits the natural unconditioned response that an animal makes following a particular stimulus (i.e. the 'unconditioned stimulus', such as the salivation response of Pavlov's dogs when presented with food). By pairing this stimulus with a different stimulus, that alone would not alone provoke the particular unconditioned response (i.e. the 'conditioned stimulus', like the sounding of a bell), the animal learns to associate the two together and thus starts to exhibit the initial unconditioned response (now the 'conditioned response') when presented with the conditioned stimulus. In other words, the animal has learnt to predict that the unconditioned stimulus must soon follow (i.e. salivate in response to a bell in anticipation of the arrival of food).

Some studies using this paradigm have replaced the conditioned stimulus with the electrical stimulation of a particular afferent sensory pathway (e.g. Matthies et al., 1986;Doyere and Laroche, 1992); the goal being to detect changes in synaptic strength as evidence that plasticity occurs with learning (i.e. the first criterion listed here). However, as pointed out by Martin and Morris (2002) this could be considered as a half-way house towards mimicry, especially if a preparation were to be developed where after conditioning, the learnt association could then be elicited by a natural sensory experience that was encoded by the identical neural pathway that was used as the electrically-induced conditioned stimulus. More recent studies have taken steps closer towards attaining mimicry. For example, McLin and colleagues were able to precipitate an unconditioned response (changes in respiration and heart rate) with electrical stimulation of the *nucleus basalis*, and when paired with the sounding of an auditory tone (the conditioned stimulus) animals could successfully learn to associate

the two, such that subsequent presentation of the tone would alone generate the conditioned response (McLin et al., 2002). They also observed receptive field plasticity in the auditory cortex similar to that observed after standard auditory conditioning (e.g. Kilgard and Merzenich, 1998) supporting the hypothesis that plasticity was the substrate for learning. The next step towards true mimicry would be to replace the auditory tone with an exogenous electrical stimulus that can accurately mimic a tone, such that the presentation of the tone itself would also provoke a conditioned response after learning and then to identify the specific synaptic changes that underlie the memory.

Although true mimicry of associative learning is yet to be achieved, it seems likely this approach will eventually enable scientists to artificially engineer or 'implant' a memory of an event that never actually occurred into the brain of an animal. Such a model would then also enable the experimenter to meet the other criteria; i.e. to detect plastic changes at the synapses required for the association, to block the acquisition of the memory by inhibiting the processes that underlie those plastic changes, and to artificially erase the memory after it is implanted by reversing the plastic changes and observing subsequent forgetting.

1.7 Cellular Processes underlying Bidirectional Synaptic Plasticity in the Hippocampus

Various distinct forms of homosynaptic LTP and LTD have now been identified throughout the brain, characterised by their diversity of their induction and expression mechanisms (Kemp and Bashir, 2001;Malenka and Bear, 2004;Lynch, 2004). However, it is the bidirectional changes in synaptic strength that can be induced at the Schaffer collateral to CA1 excitatory synapses that have received the most experimental attention. These synapses are also the main focus of the work carried out in this thesis and so a summary of the main underlying cellular processes of plasticity will be limited to this pathway.

1.7.1 LTP & LTD Induction Mechanisms

The most intensely studied forms of LTP and LTD at CA1 synapses are those induced by the respective high- and low-frequency stimulation of the Schaffer collateral axons.

Pharmacological experiments have shown that both these forms of plasticity require the activation of postsynaptic NMDA receptors (Collingridge et al., 1983;Coan et al., 1987;Bashir et al., 1990;Dudek and Bear, 1992;Mulkey and Malenka, 1992). The activation of mGlu receptors has been found to modulate LTP but they cannot underlie its induction without concurrent NMDA receptor activation (Selig et al., 1995). In contrast, NMDA receptor-independent forms of LTD can be induced at these synapses, like mGluR-mediated and endocannabinoid-mediated LTD (Malenka and Bear, 2004). However, the NMDA receptor-dependent form of LTD is of particular interest because a key protein that is modulated and underlies its expression is the same as is modulated and underlies the expression of LTP; this common substrate is the GluR1 AMPA receptor subunit (Lee et al., 2000). The activation of mGlu receptors can in some circumstances trigger the induction of a phosphatase-independent form of LTD (Oliet et al., 1997), highlighting the possibility that a diverse array of molecular pathways may be recruited that can lead to synaptic depression in the brain.

Such NMDA receptor-mediated changes in synaptic strength have received intense investigation because of the ability of the NMDA receptor to act as a molecular detector of coincident pre- and postsynaptic activity; a property that provides a potential cellular analogue of associative learning which is a key component of synaptic models of learning and memory in the brain (Bliss and Collingridge, 1993). As mentioned earlier, the key property of the NMDA receptors that underlies coincidence detection is the voltage-dependent block of the NMDA receptor ion channel by extracellular Mg^{2+}. Relief from the channel block is only achieved when the postsynaptic membrane is sufficiently depolarised; this can result from prior AMPA receptor-mediated synaptic transmission at that input, synaptic activity at nearby synapses on the same neurone, or from the occurrence of back-propagating action potentials that invade the postsynaptic dendrites (Magee and Johnston, 1997;Johnston et al., 2003). Only when synaptic transmission occurs at the same time as postsynaptic depolarisation can released glutamate activate the NMDA receptors and permit Ca^{2+} to enter the postsynaptic cell.

The signalling cascades that underlie LTP and LTD are both dependent upon this NMDA receptor-mediated influx of Ca^{2+} into the postsynaptic dendritic spine. This was initially demonstrated by adding Ca^{2+} chelators to the postsynaptic cell and observing

the subsequent block of LTP (Lynch et al., 1983;Malenka et al., 1992) and LTD induction (Mulkey and Malenka, 1992). So how are neurones able to differentiate between LTP-inducing Ca^{2+} signals and LTD-inducing Ca^{2+} signals? A popular model for the control of bidirectional plasticity has been based upon the idea that the deciding factor is the actual amount of integrated NMDA receptor activation and thus the *magnitude* of the resulting postsynaptic Ca^{2+} influx (Lisman, 1989). Numerous lines of evidence have supported this proposal (reviewed in Kemp and Bashir, 2001); for example, using the photolytic uncaging of intracellular postsynaptic Ca^{2+} it was found that LTP could be selectively triggered by a brief but high increase in Ca^{2+} concentration that mimics the Ca^{2+} rise achieved with LTP-inducing electrical stimulation; whereas, a prolonged modest rise of Ca^{2+}, as would be achieved with low-frequency LTD-inducing stimuli, could reliably induce synaptic depression (Yang et al., 1999). Strong evidence has also come from the observation that LTP-inducing stimuli can result in LTD when delivered in the presence of a low concentration of an NMDA receptor antagonist (Cummings et al., 1996;Nishiyama et al., 2000).

Recent evidence has additionally indicated that the subunit composition of the NMDA receptors may play an important role in governing the direction of synaptic plasticity (Liu et al., 2004). Liu and colleagues (2004) found that selectively blocking NR2B-containing receptors prevented LTD induction without affecting LTP production, whereas the preferential inhibition of NR2A-containing NMDA receptors abolished the induction of LTP but did not affect the ability to induce LTD. Factors that may contribute to the distinct contributions of these the NR2A- and NR2B-containing NMDA receptors and the respective induction of LTP and LTD may involve: differences in the signalling proteins that associate with their cytoplasmic tails (Husi et al., 2000;Bliss and Schoepfer, 2004); differences in their synaptic locations (NR2A-containing-receptors are primarily synaptic whereas NR2B-containing receptors are extra-synaptic; Stocca and Vicini, 1998;Tovar and Westbrook, 1999;Steigerwald et al., 2000); and the fact that the synaptic currents mediated by these receptors display distinct kinetics – the decay time constants being approximately 50 and 300 ms respectively for the NR2A-containing and NR2B-containing NMDA receptors respectively (Cull-Candy et al., 2001). Regarding this latter possibility, low-frequency stimulation might allow a temporal summation of Ca^{2+} influx via the NR2B-containing

receptors due to their slower decay kinetics and provide the Ca^{2+} stimulus required for LTD induction, whereas this would not occur with the much faster decaying responses of the NR2A-containing receptors (Bliss and Schoepfer, 2004).

Thus, specific spatial and temporal properties of the Ca^{2+} signals may also play an important role in the selective triggering of the distinct LTP and LTD signalling cascades. However, as the act of measuring Ca^{2+} (with the current methodologies available) distorts the Ca^{2+} signal itself, the crucial experiment required to determine these factors cannot be performed and it remains elusive whether it is the absolute concentration of Ca^{2+} attained, the specific spatial and temporal pattern of Ca^{2+} rises, or a combination of these factors that is important for the selective triggering of LTP and LTD (Yang et al., 1999;Cho et al., 2001;Carlisle and Kennedy, 2005). It also remains unsolved whether the activation of NMDA receptors is alone sufficient to elicit these stable long-lasting forms of plasticity or whether other factors are also necessary, such as the contemporary activation of metabotropic receptors (Naie and Manahan-Vaughan, 2005).

It is, however, now well established that the expression of LTP in CA1 neurones requires the preferential activation of Ca^{2+}-activated protein kinases, in particular the activation of CaMKII (Fukunaga and Miyamoto, 2000;Lisman et al., 2002;Rongo, 2002); whereas LTD expression is triggered via increased protein phosphatase (PP) activity, namely PP1 and PP2B (Winder and Sweatt, 2001;Mansuy, 2003). Indeed the balance between kinase and phosphatase activity is now known to be a crucial factor controlling bidirectional plasticity at numerous synapses in the brain, including the Schaffer collateral inputs to CA1. For example, injecting specific inhibitors of CaMKII into postsynaptic CA1 neurones blocks the induction of LTP (Malenka et al., 1989;Malinow et al., 1989;Otmakhov et al., 1997;Chen et al., 2001). Moreover, elevating CaMKII activity, either by injecting the kinase directly into postsynaptic neurones or by the expression of a constitutively active form of the kinase, enhances synaptic transmission in a manner that occludes the induction of further potentiation with LTP-inducing stimuli. This suggests that LTP and CaMKII enhance synaptic transmission by the same mechanism (Pettit et al., 1994;Lledo et al., 1995;Shirke and Malinow, 1997).

A wealth of evidence has similarly been gathered to support the importance of PP1 and PP2B activity for the expression of various forms of LTD (Winder and Sweatt, 2001;Mansuy, 2003;Munton et al., 2004). For instance, pharmacological inhibition of the PP2B or PP1 phosphatase activity blocks the induction of LTD induced by low-frequency stimulation or NMDA treatment in the hippocampus and the visual cortex (Mulkey et al., 1993;Kirkwood and Bear, 1994;Mulkey et al., 1994;Hodgkiss and Kelly, 1995;Torii et al., 1995;Kamal et al., 1999), and injecting active PP1 into CA1 pyramidal neurones enhances the magnitude of LTD (Morishita et al., 2001). In support of the role of PP2B, the induction of LTD by low-frequency stimulation in the visual cortex was found to cause a rapid and persistent up-regulation of PP2B activity (Yasuda et al., 2003b) and, as mentioned earlier, the genetic deletion of the PP2B regulatory subunit results in significantly reduced LTD in CA1 (Zeng et al., 2001).

1.7.2 Ca^{2+}/ Calmodulin Triggered Signalling Cascades

So how can distinct patterns of postsynaptic Ca^{2+} influx result in the preferential activation of these distinct signalling pathways? The key to this question lies in the selective activation of calmodulin (CaM)-stimulated protein kinases and phosphatases. CaM is a small (148 amino acids) and highly conserved protein that is ubiquitously expressed in all mammalian cells and is one of the principal sensors of increases in intracellular Ca^{2+} concentration and an important activator of Ca^{2+}-sensitive proteins (Chin and Means, 2000). Indeed a large number of neuronal proteins are activated by Ca^{2+}-bound CaM (Ca^{2+}/CaM); examples include: the protein phosphatase PP2B (Aramburu et al., 2000), various protein kinases, including CaMKI, CaMKII and CaMKIV (Hook and Means, 2001), the adenylyl cyclases 1 and 8 (AC1 and AC8; Westcott et al., 1979), nitric oxide synthase (Bredt and Snyder, 1990), several ion channels (including voltage-gated Ca2+ channels; Lee et al., 2003a), the plasma membrane Ca^{2+} pump (Jarrett and Penniston, 1977;Vincenzi and Larsen, 1980), IP3 receptors (Tang et al., 2001a), phosphodiesterase (Shenolikar et al., 1985) and a number of cytoskeletal proteins (Xia and Storm, 2005). Due to the large number and variety of proteins that Ca^{2+}/CaM either activates or modulates the function, this Ca^{2+} sensor essentially participates in virtually all cellular processes, from its role as a signal integrator in synaptic plasticity to cell division and motility (Xia and Storm, 2005).

The key to the controlled activation of these various molecules lies in two important facts. The first is that they show significant differences in their relative affinities for CaM (Klee et al., 1979;Klee, 1988;Maier and Bers, 2002) and the second is that the concentration of CaM in neurones is far less than the total concentration of its potential binding-partners (Black et al., 2004). Thus small Ca^{2+} influxes will generate a limited supply of Ca^{2+}/CaM and will favour the activation of the high-affinity Ca^{2+}/CaM-activated proteins, whereas large Ca^{2+} influx will enable the activation of both the low- and high-affinity Ca^{2+}/CaM-binding proteins (figure 1.2). In other words, the competition for Ca^{2+}/CaM offers a specific way in which the relative activities of PP2B and CaMKII can be correlated with the magnitude of Ca^{2+} influxes and thus the respective induction of LTD and LTP.

Indeed, PP2B exhibits one of the highest affinities for Ca^{2+}/CaM (Kd = 0.1 nM), whereas the affinity of CaMKII for Ca^{2+}/CaM is substantially less (Kd = 20-100nM; Maier and Bers, 2002). Thus a moderate influx of Ca^{2+} into the postsynaptic cell and a limited availability of Ca^{2+}/CaM, as would occur with LTD-inducing stimuli, would lead to the preferential activation of PP2B over CaMKII. Importantly, increased PP2B activity will also bring about an increase in PP1 activity through the dephosphorylation and hence inactivation of the PP1 inhibitor protein, inhibitor-1 (Lisman, 1989). These Ca^{2+}-stimulated increases in the PP1 and PP2B phosphatase activities are then thought to lead to the expression of LTD through the dephosphorylation of numerous protein substrates.

Interestingly, many of the key substrates for dephosphorylation during LTD expression are not the same substrates that are phosphorylated by CaMKII during LTP; instead they have been identified as being specific substrates for either cAMP-stimulated protein kinase (PKA) or protein kinase C (PKC; e.g. Lee et al., 1998;Kameyama et al., 1998;Hrabetova and Sacktor, 2001;van Dam et al., 2002). Thus, despite the control of LTD and LTP expression being tightly regulated by a balance between phosphatase and kinase activity and protein phosphorylation states, the molecular pathways underlying their expression are not simply mirror images of each other but involve the targeted alteration of distinct substrates.

A particularly strong case has been made for the required dephosphorylation of PKA substrates for LTD expression. Evidence for this includes the observation that postsynaptic activation of PKA can reverse previously established LTD without affecting baseline transmission (i.e. pre-LTD levels are attained but not more potentiated ones; Kameyama et al., 1998). Moreover, a PKA-dependent form of LTP in CA1 was found to be greatly enhanced in the hippocampus of transgenic mice over-expressing an autoinhibitory peptide of PP2B (Winder et al., 1998). Indeed the concerted actions of PKA and PP2B are most likely facilitated by fact that they both associate to A-kinase-anchoring protein (AKAP), a scaffolding protein that targets these enzymes to the PSD (Coghlan et al., 1995).

Furthermore, as the PP2B-PP1 protein phosphatase cascade is thought to be preferentially activated by Ca^{2+}/CaM over that of CaMKII, phosphatase activity may directly impose a negative constraint over the kinase-mediated processes that lead to synaptic potentiation (i.e. via dephosphorylation of their shared substrates; Mansuy, 2003;Ishida et al., 2003). In fact, one of the targets of PP1 activity is the CaMKII molecule itself, the dephosphorylation of which negatively regulates its activity as well as affecting various other CaMKII properties that are considered to be important for its role in synaptic potentiation, such as its subcellular location (see section 1.10.6). Another mode by which phosphatase activity may inhibit LTP induction is via the PP2B-dependent dephosphorylation of NMDA receptors; this reduces the channel open time (Shi et al., 2000) and could therefore lead to a reduced Ca^{2+} influx through the activated receptor and thereby favour LTD induction over LTP.

A large Ca^{2+} influx, on the other hand, would increase the activation of both the low- and high-affinity Ca^{2+}/CaM-binding proteins as the competition for available Ca^{2+}/CaM is decreased. Thus the relative levels of CaMKII activity would be enhanced, as well as that of the Ca^{2+}/CaM-stimulated adenylyl cyclases (ACs) which have one of the lowest affinities for Ca^{2+}/CaM (Kds > 100nM; Maier and Bers, 2002). The adenylyl cyclases catalyse the conversion of ATP to cAMP, a second messenger that in turn regulates the activity of several neuronal proteins (Ferguson and Storm, 2004) and is required for the late-, protein synthesis-dependent, phase of CA1 LTP (e.g. Frey et al., 1993;Wong et al., 1999) in addition to playing other roles during earlier phases of LTP expression. An

important target of cAMP activation is PKA. PKA activity is important for LTP induction as it phosphorylates inhibitor-1 (Huang and Glinsmann, 1976) and thus counteracts and competes with PP2B activity for this substrate. The phosphorylated form of inhibitor-1 is able to bind to and inhibit PP1 activity. Thus the higher Ca^{2+} fluxes that are necessary for LTP induction also result in the inhibition of one of the key molecules required for synaptic depression. Furthermore, as one of the targets of PP1 is CaMKII (Strack et al., 1997a), inhibition of PP1 also acts to protect the phosphorylation state of CaMKII and thus maintain the activity and potentiating actions of CaMKII within the synapse.

In support of the requirement of cAMP-dependent inhibition of PP1 for LTP induction, the blockade of LTP through PKA antagonism can be overcome by the injection of constitutively active inhibitor-1 into CA1 neurones of rat hippocampal slices, yielding LTP indistinguishable from controls (Blitzer et al., 1998). However, the injection of constitutively active inhibitor-1 did not rescue LTP inhibition from CaMKII inhibition, supporting the hypothesis that this PKA pathway uses PP1 inhibition to 'gate' CaMKII signalling for LTP induction (Blitzer et al., 1998). Furthermore, cAMP/CaMKII-dependent LTP induced by theta frequency stimulation was shown to be dependent upon PP1 inhibition and could be mimicked by CA1 injections of constitutively active inhibitor-1 (Brown et al., 2000).

The availability of cAMP is thus an important factor in the regulated inhibition of PP1. In support of a role of this pathways for endogenous learning mechanisms, spatial learning was found to up-regulate the expression of the Ca^{2+}/CaM-stimulated ACs in the CA1/CA2 region of the mouse hippocampus (Guillou et al., 1999;Mons et al., 2003). Indeed, the activity-dependent bidirectional regulation of PP1 by PP2B and PKA via control of the phosphorylation state of inhibitor-1 is considered to be a crucial element in the control of bidirectional changes in synaptic strength (Lisman and Zhabotinsky, 2001;Winder and Sweatt, 2001).

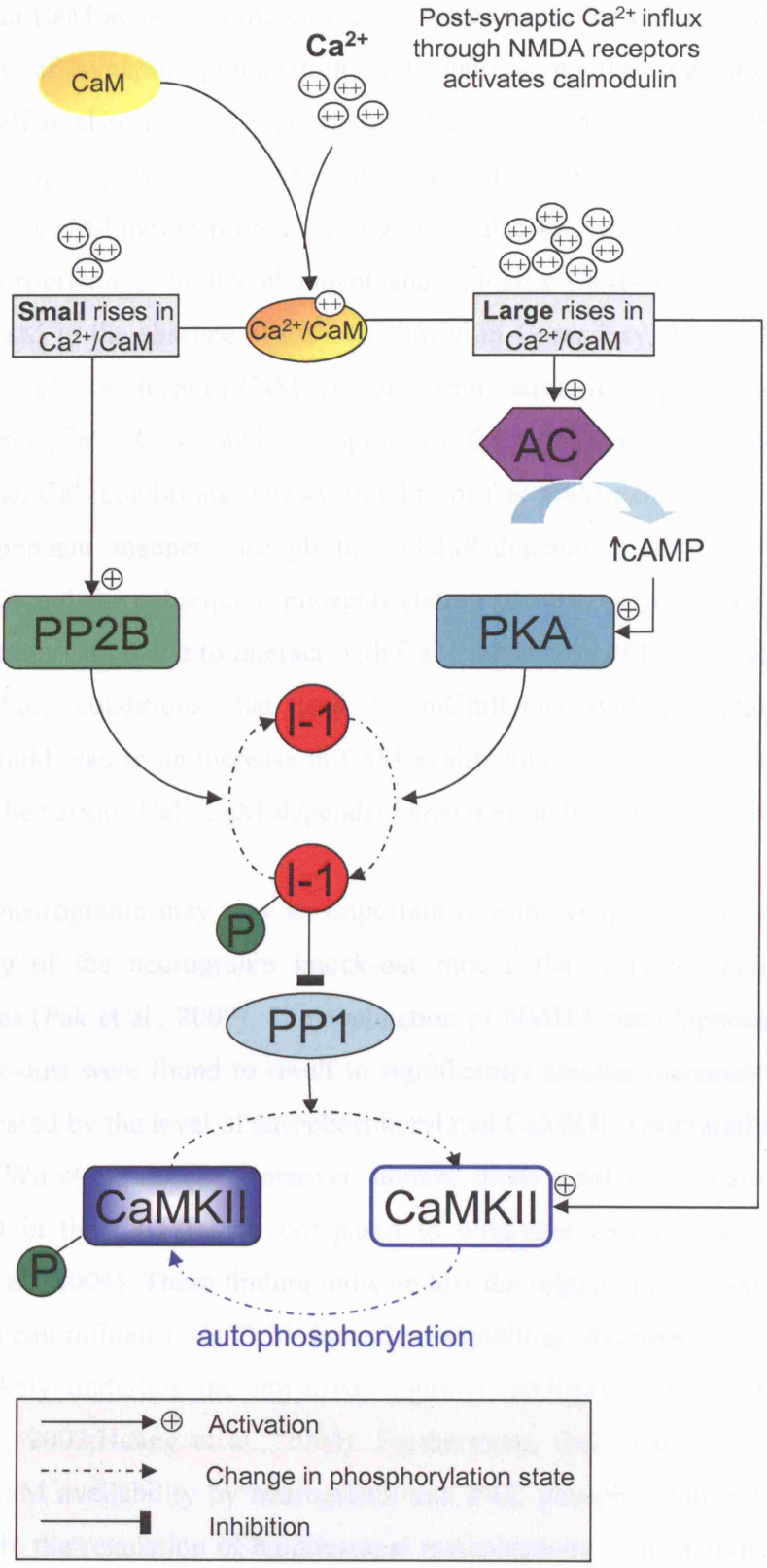
Another enzyme activated by Ca^{2+}/CaM that may also play a modulatory role in synaptic plasticity is phosphodiesterase. Phosphodiesterase is responsible for hydrolyzing cAMP and therefore exerts a negative constraint upon cAMP-stimulated pathways. Interestingly this enzyme has a high affinity for its activator (Kd = 0.1-

1nM), much greater than that for AC activation (Detre et al., 1984). Thus under conditions of low Ca^{2+} increases, the hydrolysis of cAMP by phosphodiesterase may act to restrict any PKA activity that does arise and thus favour the induction of LTD through PP1 activation and prevent moderate rises in Ca^{2+} from triggering potentiating pathways.

Figure 1.2 The Activity-Dependent Regulation of Ca^{2+}-Activated Kinases and Phosphatases.

Small rises in postsynaptic Ca^{2+} favour an increase in protein phosphatase activity over kinase activity. This is due to a) the higher affinity of PP2B for Ca^{2+}/CaM over that of CaMKII and b) the relief of PP1 inhibition via the PP2B-dependent dephosphorylation, and hence inactivation, of inhibitor 1; the endogenous inhibitor protein of the PP1. Enhanced PP1 activity will also promote the net de-phosphorylation and thus inactivation of CaMKII.

With larger rises in postsynaptic Ca^{2+} concentration and thus Ca^{2+}/CaM availability, the relative activities of the lower affinity CaM-activated enzymes like AC1 and AC8 and CaMKII will be increased. The increase in AC activity and thus cAMP levels will also promote the inhibition of PP1, and therefore the levels autophosphorylated CaMKII, due to the increased PKA-dependent phosphorylation of inhibitor 1.


Post-synaptic Ca2+ influx through NMDA receptors activates calmodulin
Ca2+
CaM
Small rises in Ca2+/CaM
Ca2+/CaM
Large rises in Ca2+/CaM
AC
↑cAMP
PP2B
PKA
I-1
I-1
P
PP1
CaMKII
CaMKII
P
autophosphorylation
Activation
Change in phosphorylation state
Inhibition

1.7.2.1 The Control of Free Calmodulin (CaM) in Neurones

Considering that CaM-activated kinases and phosphatases are essential for the induction and regulation of synaptic potentiation and depression, the regulation of CaM availability itself is also, therefore, an important factor in the control of hippocampal bidirectional synaptic plasticity. In neurones, the availability of postsynaptic CaM is regulated by the CaM-binding protein neurogranin (also known as RC3; Gerendasy et al., 1994). Neurogranin is highly abundant and contains CaM-binding domains that interact with CaM in the absence of Ca^{2+} (reviewed in Gerendasy, 1999). Neurogranin can bind to the Ca^{2+}-free form of CaM with an affinity equal to or greater to that of the Ca^{2+}-bound form ($Ca^{2+}{}_4$-CaM) and is responsible for maintaining low levels of free CaM under basal Ca^{2+} conditions. The availability of free CaM can also be enhanced in an activity-dependent manner through the mGluR-dependent activation of protein kinase C (PKC) and the subsequent phosphorylation of neurogranin (Ramakers et al., 1999) which renders it unable to interact with CaM regardless of Ca^{2+} levels (Huang et al., 1993). Thus, conditions that lead to mGluR-mediated phosphorylation of neurogranin would lead to an increase in CaM availability which may in turn promote the activity of the various Ca^{2+}/CaM-dependent enzymes under certain conditions.

Evidence that neurogranin may play an important role in synaptic plasticity has come from the study of the neurogranin knock-out mouse that exhibit impaired spatial learning abilities (Pak et al., 2000). The application of NMDA onto hippocampal slices from the knock-outs were found to result in significantly smaller increases in CaMKII activation (assessed by the level of autophosphorylated CaMKII) compared to wild-type control slices (Wu et al., 2003). Moreover, mutant slices exhibited impaired LTP and enhanced LTD in the CA1 region compared to wild-type controls (Krucker et al., 2002;Huang et al., 2004). These finding indicate that the regulation of CaM availability by neurogranin can influence Ca/CaM-dependent signalling cascades, the disruption of which most likely underlies the impaired cognitive abilities in these mutant mice (Krucker et al., 2002;Huang et al., 2004). Furthermore, these data indicate that the regulation of CaM availability by neurogranin and PKC phosphorylation may play an important role in the regulation of hippocampal metaplasticity – the activity-dependent regulation of activity thresholds required for the induction of synaptic plasticity

(Krucker et al., 2002;Huang et al., 2004).

Neurogranin is not located in nerve terminals, but instead the CaM-binding protein neuromodulin (also known as GAP-43) fulfills the presynaptic role as regulator of CaM signalling. Presynaptic targets of CaM include CaMKI and II, PP2B, the ACs 1 and 8 (and thus cAMP activated molecules, like PKA) and various cytoskeletal molecules (Mansuy, 2003;Xia and Storm, 2005). Neuromodulin is similar to neurogranin in that it can be phosphorylated by PKC (Apel et al., 1990) which can therefore provide a mechanism for the activity-dependent release of CaM. In contrast to neurogranin however, neuromodulin has a much lower affinity for CaM in the presence of free Ca^{2+}, thus Ca^{2+} influx into the presynaptic terminal itself triggers a rise in free CaM for the activation of its presynaptic binding partners. The phosphorylation of neuromodulin was found not to be required for LTP in CA1 but when it was mutated in order to mimic the behaviour of the phosphorylated form, LTP was enhanced suggesting that the PKC-dependent control of free CaM levels may be able to contribute towards synaptic plasticity (Hulo et al., 2002).

1.7.3 Expression Mechanisms Underlying NMDA Receptor-Mediated Forms of Synaptic Plasticity in CA1

As AMPA receptors are responsible for carrying the majority of the depolarising current in response to the synaptic release of glutamate, it is not surprising that increases and decreases in AMPA receptor-mediated synaptic transmission have been found to be the main way in which synapses modify their relative strengths (e.g. Bredt and Nicoll, 2003;Bear, 2003). These bidirectional changes can result from the changes in AMPA receptor function (e.g. Benke et al., 1998;Lee et al., 2000), from increases or decreases in the level of receptor expression within the PSD (Malinow and Malenka, 2002;Song and Huganir, 2002;Bredt and Nicoll, 2003;Malinow, 2003) and from presynaptic changes in neurotransmitter release at the Schaffer collateral terminals (Zakharenko et al., 2001;Emptage et al., 2003;Choi et al., 2003). Presynaptic modifications can result in changes in the probability that an action potential initiates the release of neurotransmitter or as changes that result in altered concentrations of cleft glutamate following release (e.g. Choi et al., 2000;Choi et al., 2003;Voronin and Cherubini, 2004). Unlike the well documented presynaptic locus of synaptic plasticity at some synapses,

like at the mossy fibre to CA3 synapse (e.g. Reid et al., 2004), the contribution of presynaptic changes to plasticity at CA1 inputs are only recently starting to be unraveled. It is interesting to consider that such a dual pre- and postsynaptic locus for plasticity increases a synapse's potential repertoire of activity-dependent changes that may be recruited under different conditions. It also, unfortunately, renders plasticity much more difficult to study and understand.

With regards to the postsynaptic mechanisms, much progress has been made in the last few years linking activity-dependent kinase and phosphatase activities with the regulation of AMPA receptor-mediated synaptic transmission and has provided some insight into the cellular mechanisms underlying both LTP and LTD. The cellular processes that result in *enhanced* AMPA receptor transmission (i.e. LTP) have received the most attention and are thus better understood than the processes that underlie decreases in AMPA receptor transmission. This probably reflects the earlier discovery of LTP compared to that of LTD and the overall greater interest in LTP as a phenomenon that may underlie learning and memory in the hippocampus over that of LTD. The study of LTD is also complicated by its multiple forms of induction and expression and apparent sensitivity to experimental conditions.

A summary of the key processes that lead to altered AMPA receptor function during LTP and LTD are presented. I will limit the introduction to these pathways as they underpin one of the major expression mechanisms of synaptic plasticity and AMPA receptors are a key target of both phosphatase and kinase activities. Moreover, modulation of AMPA receptor-mediated synaptic transmission is intimately linked with CaMKII activity – the mutated form of which is exploited in this thesis in order to investigate the effects of an aberrant plastic nervous system upon the properties of CA1 neurones and to understand further the nature of plasticity *in vivo*.

1.7.3.1 The Phosphorylation of Existing AMPA Receptors during LTP

As discussed above, enhanced protein kinase activity, in particular that of CaMKII, is required for the induction of LTP. It has now emerged that CaMKII may modify AMPA receptor function through at least two distinct processes; the phosphorylation of existing AMPA receptors and the targeting of additional AMPA receptors to the synaptic

membrane. Both of these activity-dependent modifications specifically involve the GluR1 AMPA receptor subunit (Song and Huganir, 2002;Sheng and Lee, 2003;Bredt and Nicoll, 2003;Malinow, 2003).

It was first demonstrated *in vitro* that CaMKII was able to phosphorylate the cytoplasmic tail of GluR1 at a serine residue of position 831 (Ser831; Mammen et al., 1997;Barria et al., 1997a). Following studies found that the phosphorylation of this residue enhanced the function of homomeric GluR1 receptors (expressed in HEK cells) by increasing the probability that the channel made transitions to its high-conductance states, increasing the overall conductance of these receptors by about 50% (Derkach et al., 1999;Derkach, 2003). This could also be mimicked by replacing Ser831 with an aspartate group, confirming that the effect was caused by the phosphorylation of this residue (Derkach et al., 1999). Importantly, AMPA receptor single channel conductances have also been found to be increased in CA1 cells following LTP induction in hippocampal slices (Benke et al., 1998) and indeed Ser381 has been specifically identified as being phosphorylated during LTP (Lee et al., 2000). Further still, a similar increase in conductance has been measured in CA1 neurones transfected with a constitutively active fluorescently-tagged form of CaMKII compared to non-transfected (i.e. non-fluorescent) nearby cells (Poncer et al., 2002).

The Ser831 residue is also a substrate of PKC (Blackstone et al., 1994). However, it was found that LTP-inducing theta-burst stimulation of Schaffer collateral axons in hippocampal slice preparations does not synergistically recruit both PKC and CaMKII to phosphorylate AMPA receptors as the LTP-induced increase in AMPA receptor phosphorylation in these neurones could be completely blocked by sole use of the CaMKII antagonist, KN-62 (Barria et al., 1997b). This study also showed that although the level of AMPA receptor phosphorylation was significantly increased at 15 and 60 minutes post LTP induction, it was unchanged at 5 minutes. This indicated that NMDA receptor-dependent phosphorylation of AMPA receptors by CaMKII could not contribute to the immediate early phase of LTP expression (Barria et al., 1997b). This was also supported by their observation that the CaMKII inhibitor KN-62 did not block the initial potentiation following LTP induction but was only inhibitory 10-15 minutes after theta-burst stimulation.

In contrast to the effects of Ser831 phosphorylation in GluR1 homomeric AMPA receptors, a recent study has unveiled evidence showing that the phosphorylation of GluR1 Ser831 within GluR1-GluR2 heteromeric AMPA receptors expressed in HEK cells does not lead to an increase in receptor conductance (Oh and Derkach, 2005). Thus, the presence of the GluR2 subunit appears to disrupt the coupling between Ser831 phosphorylation and enhanced channel function. This is an important discovery since the GluR1-containing AMPA receptors found in mature hippocampal neurones are predominately comprised of GluR1-GluR2 heterodimers (Wenthold et al., 1996). As pointed out by Oh and Derkach (2005), this does not exclude the possibility that other protein-protein interactions mediated by CaMKII activity may regulate the conductance of endogenous GluR1-GluR2 receptors in neurones. Another possibility is that changes in AMPA receptor subunit composition could occur following LTP induction such that the number of GluR2-lacking receptors are increased within synapses (Ju et al., 2004); Ser831 phosphorylation in these receptors could then contribute to enhanced AMPA receptor-mediated synaptic transmission.

Another recent study assessed the changes in AMPA receptor single channel properties following either LTP induction (100 Hz stimulation, 1 second) or increased CaMKII activity in outside-out patches that were excised from the apical dendrite of CA1 pyramidal neurones near the point of stimulation (Andrasfalvy and Magee, 2004). In accordance with Oh and Derkach (2005), they did not detect any changes to the single channel conductances of these extrasynaptic receptors, although a 2-fold increase in AMPA receptor number was found. This contrasts with the findings of Benke et al. (1998) where single channel conductances of synaptic receptors following pairing-induced LTP were enhanced and indeed sufficient to account for the increased EPSC amplitudes. One possible explanation for this discrepancy may be that the effects of CaMKII activity and LTP induction upon AMPA receptors located within dendritic spines are different to the changes occurring in the apical dendrite shaft; for example the newly inserted AMPA receptors within the dendritic shaft may serve to *supply* spinous synapses with additional receptors that when inserted may then become phosphorylated at Ser831 (Andrasfalvy and Magee, 2004). The different results might also reflect different mechanisms of potentiation that are recruited by the respective pairing- and

tetanus-induced LTP protocols that were used in the two studies.

1.7.3.2 The Insertion of Additional AMPA Receptors during LTP

The insertion of additional GluR1-contianing AMPA receptors into synapses following NMDA receptor-dependent LTP induction was first observed in organotypic slice cultures (Shi et al., 1999). This was elegantly demonstrated with fluorescence microscopy and the visualization of GluR1 GFP-tagged subunits. This laboratory went on to show that GluR1 AMPA receptor insertion was driven by CaMKII activity and that it did not involve the phosphorylation of GluR1 at Ser831 (Hayashi et al., 2000). Thus the CaMKII-induced delivery of additional AMPA receptors to the synapse reflects a distinct mechanism of synaptic potentiation to that involved in the modification of existing AMPA receptors. In contrast, the phosphorylation at a second site on the cytoplasmic tail of GluR1 receptors, Ser845, has been reported as necessary, although not sufficient, for the synaptic insertion of additional AMPA receptors (Esteban et al., 2003). The Ser845 site is a substrate for PKA activity (Roche et al., 1996) and thus implicates a second important "gating" role of PKA in LTP induction (Malinow, 2003). Phosphorylation of Ser845 has been shown to accompany the surface reinsertion of AMPA receptors (Ehlers, 2000) and follows LTP induction following prior LTD (Lee et al., 2000).

The mechanism by which CaMKII underlies the activity-dependent insertion of additional AMPA receptors is yet to be resolved. However, it is evident that this mode of enhanced AMPA receptor function is again dependent upon the properties of the GluR1 subunit cytoplasmic tail. Indeed, studies of AMPA receptor trafficking in the brain have now revealed that at least two distinct trafficking processes exist for the synaptic delivery of AMPA receptors, one that is dependent upon specific protein interactions formed with the GluR1 cytoplasmic tail and one that is dependent upon interactions with GluR2 cytoplasmic tail (Song and Huganir, 2002;Bredt and Nicoll, 2003;Malinow, 2003).

In the hippocampus, AMPA receptors are primarily formed either of paired GluR1-GluR2 dimers, or of paired GluR2-GluR3 dimers. The GluR1-GluR2 AMPA receptors are delivered to synapses in an activity-dependent manner that requires the actions of

CaMKII and serves to increase AMPA receptor synaptic responses (Hayashi et al., 2000;Andrasfalvy and Magee, 2004)); whereas GluR2-GluR3 containing receptors are delivered in an activity-independent manner and requires the actions of the ATPase NSF (N-ethylmaleimide-sensitive fusion protein; (N-ethylmaleimide-sensitive fusion protein; Nishimune et al., 1998;Song et al., 1998;Lüscher et al., 1999).

Importantly, the specific protein interactions made by the cytoplasmic tail of the GluR1 subunit that underlie receptor targeting to the synapse are dominant over those that are made with the short cytoplasmic tails of GluR2 subunit (Shi et al., 2001). Studies that interfere with the binding of proteins to these subunit cytoplasmic tails have found that the synaptic insertion of GluR1-GluR2 AMPA receptors is specifically instigated by the interactions made by the GluR1 subunit and not by its fellow GluR2 subunit; whereas the rapid and continual cycling GluR2-GluR3 AMPA receptors is governed by the interactions made by the cytoplasmic tail of GluR2 (Shi et al., 2001;Passafaro et al., 2001;Piccini and Malinow, 2002). This latter process, occurring in the absence of synaptic activity, is thought to be important for replacement of existing AMPA receptors within a synapse (Shi et al., 2001). For example, the study by Shi and colleagues (2001) found that GluR2-homomers are not driven into silent synapses, supporting their observations that GluR2 receptors only exchange with AMPA receptors already present at the synapse. This process may also be important for LTP, however, in terms of the maintenance of enhanced transmission, as it provides a mechanism by which increased AMPA receptor numbers can be maintained in the face of protein turnover (Zhu et al., 2000;Shi et al., 2001).

The mechanism by which CaMKII activity initiates the trafficking of GluR1-containing AMPA receptors is yet be to unraveled; however, as the direct phosphorylation of the GluR1 tail by CaMKII does not seem to be required for its insertion (Hayashi et al., 2000), it is generally hypothesised that the CaMKII-dependent phosphorylation of some other protein is required to interact with GluR1 and underlie its targeting to the synapse during LTP. One candidate molecule is stargazin, a transmembrane protein that interacts with all of the AMPA receptor subunits and is involved in both the surface expression and synaptic clustering of AMPA receptors (Chen et al., 2000b). Recent work has indicted that stargazing becomes phosphorylated at a number of sites during NMDA

receptor-mediated LTP induction by CaMKII and PKC and that phosphorylation of these sites is required for the LTP expression (Tomita et al., 2005). This study also found that dephosphorylation of these sites by PP1 and PP2B was required for LTD induction, implicating an important role of stargazing in bidirectional changes in synaptic strength via AMPA receptor trafficking. How stargazing phosphorylation promotes synaptic trafficking, however, remains to be discovered.

Some insight into identifying the distinct trafficking processes of GluR1 and GluR2 has been gained by recognising that the long cytoplasmic tail of GluR1 terminates with a sequence that conforms to the consensus motif for type-I PDZ domain interactions, whereas the short cytoplasmic tail of GluR2 terminates with the consensus for type II PDZ interactions (Bredt and Nicoll, 2003). PDZ domains are well characterised, highly abundant, protein sequences that bring about the binding of two, often non-similar, proteins (Sheng and Sala, 2001). Synaptic proteins commonly contain multiple PDZ domains and are thus able to bring several proteins together which can underlie the formation of large protein complexes enabling functions that include receptor clustering and the association of receptors to downstream signalling enzymes (Sheng and Sala, 2001). It is thought that such associations are likely to hold the key to the distinct regulation of GluR1- and GluR2-mediated trafficking mechanisms. For a review of the possible protein interactions that may underlie these distinct trafficking mechanisms, see Bredt and Nicoll (2003).

1.7.3.3 CaMKII as a Potential Link in AMPA Receptor Anchoring at the Synapse?

Another role of CaMKII has recently been suggested whereby CaMKII could act as a physical link to create a synaptic slot able to receive the newly inserted AMPA receptors (Lisman and Zhabotinsky 2001). This theoretical process could provide the mechanism for the activity-dependent insertion of GluR1-containing AMPA receptors into synapses or represent a third way by which CaMKII contributes to enhanced AMPA receptor synaptic transmission.

Lisman and Zhabotinsky propose that the NMDA receptor (that is present at all excitatory CA1 inputs; Takumi et al., 1999) and its associated proteins (like PSD-95 - required for the synaptic clustering of AMPA receptors; Chen et al., 2000b;Schnell et

al., 2002) represent the 'core' components of a synapse and that the recruitment of 'variable' components of a synapse, that include the AMPA receptors and their associated 'slot' or 'linker' proteins, occurs in an activity-dependent manner and requires the physical binding of CaMKII to the NMDA receptor.

A key aspect of this hypothesis has been based upon the now well accepted phenomenon whereby NMDA receptor activation can initiate the translocation of activated CaMKII to postsynaptic sites via a Ca^{2+}-dependent process (Shen and Meyer, 1999); see section 1.10.6). Furthermore a wealth of biochemical data exists to support the notion that when targeted to the PSD, CaMKII does indeed bind to the cytosolic C-termini of NMDA receptor subunits (Gardoni et al., 1998;Strack and Colbran, 1998;Leonard et al., 1999;Bayer et al., 2001).

The proteins put forward by Lisman and Zhabotinsky to associate and form these theoretical slots required for the insertion of AMPA receptors are all chosen based upon identified protein-protein interactions. A representation of how they may be able to associate and anchor AMPA receptors within the synapse is presented in figure 1.3. In summary, they have suggested that in addition to associating with the cytoplasmic tail of an NMDA receptor, the α subunit of CaMKII would also bind directly with α-actinin that in turn binds to actin. They propose that the role of actin is to form a binding site for protein 4.1 which in turn forms a complex with SAP-97; both protein 4.1 and SAP-97 can bind directly to the cytoplasmic tail of GluR1. Note, no physical evidence of this speculative receptor complex has yet been gathered.

Protein 4.1 forms a non-PDZ association with the membrane proximal region of the cytoplasmic tail of GluR1 (and GluR4) subunits (Shen et al., 2000b;Coleman et al., 2003) and has been found to associate with GluR1 *in vivo* and to colocalise with AMPA receptors at excitatory synapses (Shen et al., 2000b). Moreover, the disruption of either actin filaments or the interaction between GluR1 and protein 4.1 were both found to decrease the surface expression of GluR1 in heterologous cells. Similarly, the disruption of actin filaments in cultured cortical neurons was also seen to dramatically reduce the level of surface AMPA receptors, supporting the proposal that protein 4.1 may form a link between synaptic AMPA receptors and the actin cytoskeleton.

SAP-97, on the other hand, is the only protein so far identified to directly associate with the PDZ domain of the GluR1 subunit (Leonard et al., 1998;Cai et al., 2002), although no evidence has yet been presented that directly indicates that this protein is required for either AMPA receptor trafficking or anchoring.

Importantly, neither protein 4.1 nor SAP27 associate with GluR2 subunits or their binding proteins – GRIP (glutamate receptor interacting protein) and ABP (AMPA receptor binding protein; Dong et al., 1997;Osten et al., 2000) – and this could explain the specific synaptic recruitment of GluR1-containing AMPA receptors during the induction of LTP. This structural theory is supported by reported studies that have shown that when any of these particular binding interactions are disrupted, LTP is blocked (see Lisman and Zhabotinsky, 2001). For example, actin de-polymerisation reduces basal AMPA-mediated transmission (Zhou et al., 2001) but has no effect upon NMDA conductance (Kim and Lisman, 1999). Furthermore, Lisman and Zhabotinsky refer to studies that have shown that CaMKII, actin, α-actinin and AMPA receptors all dissociate from synaptic puncta when actin is depolymerised; consistent with the idea of these molecules being a 'variable' component of the synapse. In contrast, NMDA receptors and PSD95 do not dissociate from the synaptic puncta (Allison et al., 1998;Allison et al., 2000) in accordance with their proposed role as 'core' synaptic components. These findings support the view of Lisman and Zhabotinsky that GluR1-containing AMPA receptors, but not NMDA receptors, may be anchored into the synapse by a linkage that is dependant upon the activity-dependent delivery of αCaMKII

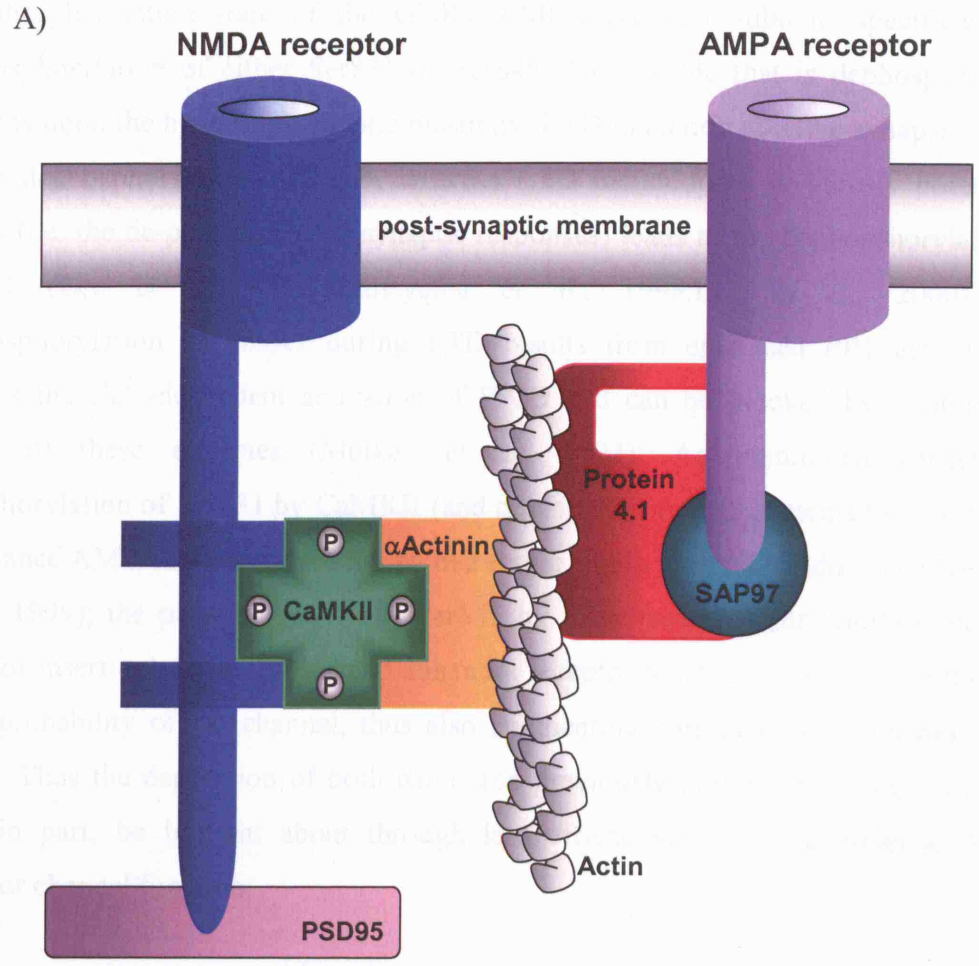
Figure 1.3 The Theoretical CaMKII Link in Synaptic AMPA Receptor Anchoring.

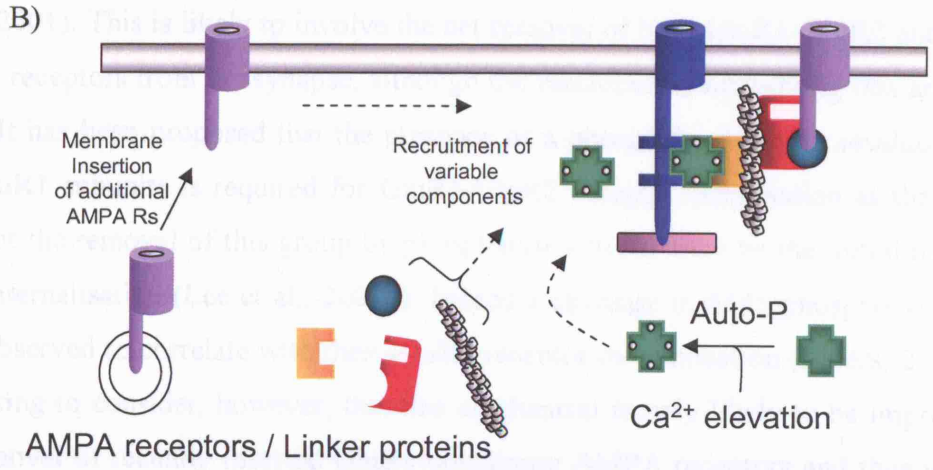
A) This schematic demonstrates the putative role, as proposed by Lisman and Zhabotinsky (2001), of CaMKII in AMPA receptor anchoring at the synapse. All of the binding interactions indicated have been demonstrated experimentally (discussed in section 1.7.3.3).

B) This cartoon presents a process by which activated CaMKII could form binding sites for the synaptic insertion of additional AMPA receptors. The autophosphorylated CaMKII binds tightly to the cytoplasmic tail of an NMDA receptor which in turn permits a chain of non-covalent protein associations to take place between some of the 'variable' components of the PSD (α-actinin, F-actin, protein 4.1 and SAP-97). This putative complex of assembled proteins is proposed to form a synaptic 'slot' that is able to receive and anchor an additional AMPA receptor within the PSD.

This theoretical process has been proposed to contribute to the CaMKII-dependent expression of LTP (Lisman and Zhabotinsky, 2001).

Adapted from Lisman and Zhabotinsky, 2001.


A)
NMDA receptor
AMPA receptor
post-synaptic membrane
Protein 4.1
SAP97
αActinin
CaMKII
P
Actin
PSD95


B)
Membrane Insertion of additional AMPA Rs
Recruitment of variable components
Auto-P
Ca2+ elevation
AMPA receptors / Linker proteins

1.7.3.4 Altered AMPA Receptor-Mediated Transmission during LTD

The depression of AMPA receptor responses has also been correlated with changes in the phosphorylation state of the GluR1 AMPA receptor subunit, specifically the dephosphorylation of either Ser831 or Ser845. The residue that is dephosphorylated depends upon the history of synaptic plasticity. LTD induction at naïve synapses results in the dephosphorylation of S845, whereas LTD induction at previously potentiated inputs (i.e. the de-potentiation of synaptic responses) leads to the dephosphorylation of Ser831 (Lee et al., 1998;Kameyama et al., 1998;Lee et al., 2000). The dephosphorylation of GluR1 during LTD results from enhanced PP1 activity that follows the Ca^{2+}-dependent activation of PP2B and can be blocked by inhibition of either of these enzymes (Mulkey et al., 1994). As mentioned earlier, the phosphorylation of Ser831 by CaMKII (and potentially of other proteins too) is thought to enhance AMPA receptor function by increasing single channel conductance (Derkach et al., 1999); the phosphorylation of Ser845 by PKA (required for GluR1-conatining receptor insertion) on the other hand enhances receptor function by increasing the peak open probability of the channel, thus also augmenting current transfer (Banke et al., 2000). Thus the depression of both naïve and previously potentiated synapses can, at least in part, be brought about through less efficacious GluR1-containing AMPA receptor channel function.

A number of studies have also provided evidence that the endocytosis of synaptic AMPA receptors is an important process underlying LTD expression (e.g. Kandler et al., 1998;Carroll et al., 1999;Lüthi et al., 1999;Beattie et al., 2000;Man et al., 2000;Zhou et al., 2001). This is likely to involve the net removal of both GluR1-GluR2 and GluR2-GluR3 receptors from the synapse, although the mechanisms underlying this are not yet clear. It has been proposed that the presence of a phosphate group on residue S845 of the GluR1 subunits is required for GluR1-GluR2 receptor stabilisation at the synapse and that the removal of this group by phosphatase activity may be the initial trigger for their internalisation (Lee et al., 2003b). Indeed a decrease in S845 phosphorylation has been observed to correlate with their GluR1 receptor internalisation (Ehlers, 2000). It is interesting to consider, however, that this mechanism is only likely to be important for the removal of recently inserted GluR1-containing AMPA receptors and thus implies a mechanism underlying the de-potentiation of only recently potentiated synapses (i.e.

before the receptor has been replaced by a GluR2-GluR3 receptor). The phosphate at S845 may be important therefore in terms of retaining a recently inserted receptor and be a signal to a potentiated synapse that phosphatase activity is still dominated over by enhanced kinase, in this case PKA, activity. A mechanism for the removal of GluR1-containing receptors has been suggested to involve the triggered depolymerisation of actin which could allow the receptors to segregate away from NMDA receptors within the PSD and diffuse to perisynaptic sites where they would then undergo internalisation by endocytosis (Zhou et al., 2001).

A more recent study has provided evidence indicating that the inducible net removal of synaptic AMPA receptors may not necessarily be caused by a triggered increase in receptor endocytosis rates but that it is the result of a decrease in the recycling rates of GluR2-containing receptors back to the membrane (Lee et al., 2004); in other words, a decrease in the rate of continuous GluR2-GluR3 receptor insertion. This study also gathered evidence indicating that LTD-inducing levels of NMDA receptor activation could trigger an increase in the degradation of AMPA receptors by diverted them away from their normal recycling pathways and towards lysosomes. As the authors point out, the cycling pool of AMPA receptors is likely to be shared by a population of synapses, so this activity-dependent decrease in AMPA receptor protein levels may also contribute towards the heterosynaptic nature of some forms of LTD.

1.8 Anatomical Correlates of Synaptic Plasticity and Memory

The functional rewiring of synaptic circuits in the brain that is thought to underlie learning and memory may also be accompanied by forms of structural plasticity; including changes in synapse and dendritic spine morphology and/or number (e.g. Lamprecht and Le Doux, 2004;Segal, 2005). However, the nature and degree of such changes that may take place physiologically and the significance of such changes is not yet well understood.

The majority of recent investigations into the structural correlates of learning and memory have primarily focused upon the plasticity of dendritic spines, the small finger-like protrusions that arise from neuronal dendrites and receive the majority of excitatory synapses in the brain (Gray, 1959). There are two main reasons for this. First of all, a

renewed interest into dendritic spine morphology and plasticity has ensued after the discovery that these microstructures act as unique chemical compartments that due to their small volume and physical segregation from the parent dendrite can accumulate high concentrations of Ca^{2+} following synaptic stimulation (Guthrie et al., 1991). As Ca^{2+} dynamics are implicitly involved in the modification of synaptic function, the plasticity of spine morphology is likely to play an important role in both the regulation and interpretation of incoming Ca^{2+} signals (e.g. Korkotian and Segal, 2000;Noguchi et al., 2005;Hayashi and Majewska, 2005). The second reason is due to the advance of confocal microscopy imaging techniques that have allowed the high-resolution visualization of dendritic spines in living tissue, thus the structural features of the spines within a particular region of dendrite can be studied both before and after a conditioning treatment in order to directly characterise changes in morphology in correlation with changes in synaptic transmission.

Even before the revolution of live confocal microscopy and the ability to visualize the plastic capabilities of dendritic spines directly, other lines of evidence had suggested that spines were not 'hard-wired' elements of the nervous system but that they could undergo plasticity in both shape and number. For example, it was found that exposing rats to learning environments resulted in increased spine numbers in the cerebral cortex (Globus et al., 1973); syndromes of mental retardation in humans were found to correlate with various changes in dendritic spine morphology (reviewed in Fiala et al., 2002); and dramatic changes in spine densities were found to occur during the oestrus cycle in rats (Woolley et al., 1990) and during hibernation in squirrels (Popov et al., 1992). The rest of this introduction section will present a brief overview of some aspects of dendritic spine structural plasticity that may occur in the brain and have been associated with either synaptic plasticity or learning experiences.

1.8.1 Spine Motility

One of the first highly significant findings revealed as a consequence of the advances in confocal microscopy and fluorescence-labelling techniques was that dendritic spines in cultured hippocampal neurones could undergo rapid and continuous size and shape changes over a time-scale of seconds and that the basis of this phenomena was due to the presence of actin within the spine heads (Fischer et al., 1998). These findings were

pivotal in recognising that changes to the structure of dendritic spines could play an important role in synaptic plasticity. Importantly, evidence has also been presented showing that highly motile spines can be innervated by a presynaptic terminal (Dunaevsky et al., 2001); thus spine motility is not simply a characteristic of non-innervated spines searching for a presynaptic partner, as may be the case for filopodia (Dailey and Smith, 1996), but motility can be considered in terms of its potential to affect the function of synaptic connections in the brain.

Spine motility ceases with the application of an AMPA receptor agonist (Fischer et al., 2000) and is enhanced by the blockade of presynaptic activity by TTX (Korkotian and Segal, 2001); thus the motile characteristics of spines appear to be inversely related to the level of synaptic activity (Korkotian and Segal, 2001). One mechanism that may control activity-dependent spine motility may involve αN-catenin, a molecule that forms a link between cadherin adhesion receptors at the synapse and the actin cytoskeleton within the spine head. The accumulation of αN-catenin within the spine head was found to be activity-dependent and associated with enhanced spine stability, while synapses that lacked αN-catenin exhibited enhanced motility (Abe et al., 2004).

Importantly, *in vivo* confocal microscopy has also demonstrated that the dendritic spines of cortical neurones can also exhibit high levels of motility in the intact living brain (Lendvai et al., 2000). The function of such dynamic behaviour is still not clear albeit heavily debated (Bonhoeffer and Yuste, 2002;Matus, 2005). Interestingly, the level of spine motility within cortical neurones appears to decrease with age (Lendvai et al., 2000;Korkotian and Segal, 2001), and it has been suggested that this decrease may be associated with changes in the level and types of plasticity that occur within cortical networks through development (Lippman and Dunaevsky, 2005).

It was recently suggested that spine motility may influence the diffusion of proteins within the plasma membrane into the spine head (Richards et al., 2004). The control of spine movements may be involved therefore in the fast redistribution of receptors proteins from non-synaptic to synaptic sites, as is thought to occur for the activity-dependent synaptic accumulation of GluR1-containing AMPA receptors during LTP expression (Choquet and Triller, 2003). It has also been suggested that rapid spine

motility could even affect the time course of Ca^{2+} dynamics in the dendritic spine and parent dendrite and for this reason play a role in the induction of synaptic plasticity (Holcman et al., 2004).

1.8.2 Structural Correlates of Synaptic Potentiation and Depression

Various studies have now gathered evidence correlating changes in dendritic spine morphology with changes in synaptic strengths. For instance, several independent groups have found that the delivery of plasticity-inducing levels of synaptic stimulation can induce the rapid expansion of spine head size (Matsuzaki et al., 2004;Okamoto et al., 2004;Otmakhov et al., 2004;Zhou et al., 2004;Lang et al., 2004). For example, Matsuzaki et al. (2004) observed that the repetitive quantum-like uncaging of glutamate near to dendritic spines of cortical pyramidal neurones *in vitro*, led to a 3-fold increase in spine volume within 2-4 minutes of stimulation, which then fell to a 20-30% increase in spine volume 20-40 minutes after stimulation. This persistent increase in spine head volume was found to be dependent upon CaMKII activity and also correlated with increases in their synaptic responses to uncaged glutamate suggesting that the spine shape changes are indeed associated with enhanced synaptic function. The activity-dependent enlargement of spine heads is also consistent with the LTP-associated increases in PSD dimensions that have been observed using electron microscopy (Fifkova and Anderson, 1981;Desmond and Levy, 1986) and with the noted correlation between spine and PSD size and concentration of glutamate receptors (Harris and Stevens, 1989;Nusser et al., 1998;Matsuzaki et al., 2001). Interestingly Matsuzaki et al. found that only small spines exhibited this structural and synaptic plasticity whereas no persistent changes were observed in large spines after treatment. Another study reported a slow (20-60 minutes) and small (16-33%) increase in the diameter of spine heads after high frequency stimulation and the potentiation of synaptic responses (Zhou et al., 2004). Moreover this study also showed that the induction of LTD by low-frequency stimulation led to a decrease in spine head dimensions, showing for the first time symmetry between the plasticity of synaptic responses and dendritic spine structure.

Evidence that such bidirectional changes are dependent upon actin dynamics within the spine heads was provided by an elegant investigation performed by Okamoto and colleagues (2004). This group developed a novel technique using fluorescence

resonance energy transfer (FRET) to monitor the equilibrium of actin between its filamentous and globular form (F- and G-actin respectively). They found that increases in spine head size with high-frequency stimulation were associated with a rapid (within 20 seconds) and persistent (at least 20 minutes) shift in the actin equilibrium towards F-actin indicating enhanced actin polymerisation. Low-frequency stimulation, on the other hand, caused spine shrinkage and a shift towards G-actin indicative of net depolymerisation. They found that changes in spine size and actin dynamics were dependent upon NMDA receptor activation and that the pharmacological induction of these two processes also mimicked the structural changes observed with stimulation protocols, together supporting the notion that actin dynamics are likely to underlie the activity-dependent plasticity of spine morphology. Okamoto et al. suggest that changes in the levels of F-actin may lead to plasticity in synaptic efficacy by modulating the capacity of actin filaments to act as a scaffold for postsynaptic proteins that modulate synaptic efficacy (such as CaMKII or the PP1-binding protein spinophilin). This agrees with the previous finding that the induction of hippocampal LTP *in vivo* is accompanied by an increase in the F-actin content within dendritic spines (Fukazawa' et al., 2003). However, as drugs that inhibit actin polymerisation block the late but not early phase of LTP, the importance of such a role of actin polymerisation appears to be unnecessary for the induction and early expression of LTP (Krucker et al., 2000;Fukazawa' et al., 2003). Nevertheless, the finding that increases in actin polymerisation are required for the persistence of enhanced synaptic strengths supports the functional significance of such structure correlates of synaptic plasticity.

In addition to the morphological changes found in existing spines, the induction of LTP has also been associated with the formation of new spines and filopodia in organotypic hippocampal slice cultures (Maletic-Savatic et al., 1999;Engert and Bonhoeffer, 1999;Toni et al., 1999;Nägerl et al., 2004), and the induction of LTD had been linked to spine retraction (Nägerl et al., 2004). For example, Engert and Bonhoeffer (1999) found that LTP-producing stimuli induced the appearance of approximately 6 new spines per 100 μm of dendrite within the stimulated region, while no significant growth occurred in control non-stimulated regions of the same dendrite or in slices where LTP was blocked. Similar results were also found by Jourdain et al. (2003) in acute brain slices, who additionally presented evidence indicating that CaMKII may play a crucial role in

the activity-dependent growth of filopodia and new spines. These phenomena were also observed in dissociated hippocampal cultures where it was shown that new spines contained glutamate receptors, were innervated by a presynaptic terminal and that their formation was dependent upon protein synthesis (Goldin et al., 2001;Goldin and Segal, 2003). Moreover, *in vivo* confocal microscopy in the mouse somatosensory cortex followed by electron microscopy of the imaged dendritic segments has revealed that the appearance of new spines was associated with synapse formation, while spine retraction correlated with synapse elimination (Trachtenberg et al., 2002).

The idea that the activity-dependent growth of new spines and synaptic contacts may provide a structural correlate of enhanced synaptic strength of specific synaptic pathways in the brain is also strengthened by the findings of Toni et al. (1999); they found that the number of presynaptic terminals that formed synapses with 2 or more dendritic spines was significantly increased in the activated pathways following LTP induction. Moreover, 3-dimensional reconstruction of the electron-micrographs revealed that these spines arose from the same postsynaptic dendrite. Thus the formation of new distinct synaptic contacts between cells may underlie the long-term maintenance of enhanced synaptic strengths in some pathways following LTP induction.

A consistent finding between all the aforementioned studies is that the formation of new spines is a slow process, with new spines not appearing before 30-60 minutes after stimulation. As yet, a link between the dynamics of spine motility and the activity-dependent formation of new synaptic contacts is missing, although it seems likely these phenomena would be intimately associated.

1.8.3 Spine Plasticity and Learning

1.8.3.1 Experience-Dependent Changes in Spine Motility

The level of motility in dendritic spines has itself also been linked with experience-dependent plasticity in the brain. In particular, confocal imaging *in vivo* has linked the level of spine motility with experience-dependent plasticity within the sensory cortex (Lendvai et al., 2000). This study imaged the dendritic spines and filopodia on pyramidal neurones within the somatosensory barrel cortex in developing rat using

time-lapse confocal microscopy and revealed that such protrusions could appear and disappear as well as undergo significant shape and length changes over a time course of tens of minutes. Indeed a surprisingly high proportion of protrusions (2-10%) would appear or disappear during a 90 minute observation period. Lendvai et al. showed that deprivation of sensory input to these neurones induced by whisker trimming resulted in a marked reduction (~ 40%) in the level of protrusive motility. However, they found that the sensitivity of motility to experience only occurred during a critical period of cortical development (postnatal days 11-13 – the period when sensory input from the whiskers profoundly influences the final organisation and tuning of sensory maps within the barrel cortex) as whisker trimming had no effect upon control levels of motility in either younger (8-10 day old) or older (14-16 day old) animals. Surprisingly, whisker trimming did not affect the average shape, length or density of either spines or filopodia indicating that strong homeostatic processes must be in place keeping spine densities constant despite changes in sensory input. These data suggest that sensory input to the cortical whisker barrels during a critical period of development is required to drive the motility of dendritic protrusions, albeit without affecting their density, and that this turnover of dendritic spines may be associated with the processes that underlie the formation of functional somatosensory synaptic circuitry.

In contrast to the above findings in the barrel cortex, sensory deprivation to the visual cortex in mice (achieved via the suturing and sealing of the eyelids before eye-opening) resulted in the *up-regulation* of spine motility in layer 5 neurones of the primary visual cortex during the peak (postnatal day 28) of the critical period (postnatal days 19-35), while no differences were observed at the start or end of this period (Majewska and Sur, 2003). Again, no changes in spine density, length or shape were induced by this procedure (Majewska and Sur, 2003). Despite the discrepancy, both of these studies support the hypothesis that spine motility is a phenomenon that can be regulated by sensory experience and is, therefore, likely to play an import role in development of cortical networks in the brain.

1.8.3.2 Learning and Changes in Spine Density

In contrast to the somatosensory and visual cortices in neonatal and juvenile animals where structural plasticity was not associated with changes in spine density, various

studies of learning and memory have presented evidence supporting the hypothesis that such cognitive processes may be associated with increases in the density of dendritic spines. For example, olfactory learning in rats was found to correlate with a 21% increases in spine density within the apical dendrites of CA1 neurones in the hippocampus and a 15% increase in layer II pyramidal neurones of the olfactory (piriform) cortex (Knafo et al., 2001;Knafo et al., 2004). No changes were found within the basal dendrites of these neurones and increases were observed 3 days but not 1 day after training completion (which takes 6-8 days). An astonishing 39% increase in CA1 spine densities has also been observed after eye-blink trace conditioning in the rat, although in contrast to olfactory learning, these changes were specific to the basal dendrites and were present already 24 hours after conditioning (Leuner et al., 2003). Similarly, training rats in complex environments to search for food and water over a period of 2-3 weeks was found to induce specific increases in spine densities within the basal dendrites of CA1 neurones (10%; Moser et al., 1994b;Moser et al., 1997). Note, however, that exposing mice to complex environments (without the requirement to hunt for sustenance) has been found to increase spine as well as synapse densities within CA1 apical dendrites (33% and 22% respective increases; Rampon et al., 2000a).

Learning-induced increases in spine density have also been identified on granule cells of the dentate gyrus. Spatial learning in the Morris water maze and the acquisition of a passive avoidance response were both found to correlate with increased dendritic spine numbers on dentate granule cells assessed 6 hours after training (O'Malley et al., 1998;O'Malley et al., 2000). In both incidences however, these increases were only transient as no changes were detected when assessed 72 hours after training. O'Malley and colleagues proposed that the transient changes in spine number may be specific to the process of memory consolidation in the dentate gyrus. They suggest that increased spine densities may reflect an increase in synapse turnover rate and correspond to the changes in connectivity associated with the processing of information for long-term storage (O'Malley et al., 2000). This hypothesis was supported by a recent ultrastructural investigation within the molecular layer of the rat dentate gyrus where learning in the Morris water maze was associated with an increase in the density of axospinous synapses at 9 hours but not at 3 or 24 hours after training (Eyre et al., 2003). The 9 hour post training increases in synapse density were also accompanied by

transient decreases in both mean synaptic height and area of postsynaptic density. No such changes were observed in exercise-matched control rats indicating that the transient synaptic changes in the dentate gyrus are most likely to be specifically related to processes involved in spatial memory formation (Eyre et al., 2003).

Studies in birds on the other hand have correlated the densities of dendritic spines more directly with memory capacity. For example, a recent study of Eastern marsh wrens found that the greater the repertoire of songs learnt by the birds the greater the density of dendritic spines within the telencephalic song control nucleus (Airey et al., 2000); the density of dendritic spines was on average 36% greater in birds that leant 36-47 songs compared to those that had learnt only 5 or 6 songs.

In summary, different learning paradigms, memory systems and cell types have all been shown to exhibit changes in spine densities following a learning experience even though the magnitudes and the time-courses of the changes have been highly variable. These various reports support, therefore, the hypothesis that the structural plasticity of dendritic spines may play a role in learning and memory formation. However, the functional significance of such changes remains elusive (discussed in: Leuner and Shors, 2004;Segal, 2005). It may be that the new spines are themselves correlates of newly laid synaptic connections and memories, although the scale of some of the changes noted makes this interpretation seem unlikely. Perhaps a more convincing hypothesis would be that increased spine numbers, usually observed with some delay after the learning experience, reflect the transition of a neurone into an altered state of excitability that is involved in the processing of information within synaptic networks, the possible rearrangement of synaptic connections and the consolidation of memory; i.e. if this were the case, changes in spine number (or motility) would correlate to the learning and memory processes but not underlie a correlate of a memory trace itself.

1.9 The CaMKII^{T286A} Transgenic Mouse as a Tool for Detecting Evidence of Endogenous Plasticity in the Hippocampus

As discussed above, a wealth of data has been gathered over the past decades supporting the hypothesis that changes in synaptic efficacy and neuronal structure can occur in the brain and that such phenomena are required for the hippocampal processes that

underlie hippocampal-dependent learning and memory. The use of molecular genetic technology has played a particularly important role in linking the function of specific molecules (often identified first *in vitro* to be necessary for experimental forms of plasticity) with their potential roles in the endogenous mechanisms that underpin learning and memory.

As mentioned earlier, the generation of the αCaMKII knockout mouse was the first of such studies to present strong evidence supporting a link between the underlying expression mechanisms of LTP with cognition in behaving animals. The mutant mice showed significant impairments in LTP expression within the CA1 region of the hippocampus and were severely impaired in tests of hippocampal-dependent spatial-reference memory (Silva et al., 1992a;Silva et al., 1992b;Elgersma et al., 2002). Some learning could be observed in these mice after extended periods of training, thought to be achieved through the compensatory up-regulation of βCaMKII translocation to activated synapses (Elgersma et al., 2002).

Since this pivotal finding, a number of other CaMKII transgenic mice have been generated where rather than knocking-out CaMKII subunits and thus simply down-regulating kinase activity, specific properties of the kinase have been altered (Mayford et al., 1995;Mayford et al., 1996;Giese et al., 1998;Elgersma et al., 2002;Miller et al., 2002;Bejar et al., 2002). These studies, in parallel with the growing depth of understanding regarding CaMKII properties and functions within neurones, have added considerable strength to the view that intact CaMKII function is a necessary component of the processes that underlie hippocampal-dependent forms of learning and memory; thus supporting the hypothesis that the role this kinase plays in experimental forms of synaptic plasticity, i.e. LTP, may also underlie its cognitive functions in the behaving animal (reviewed in: Lisman et al., 2002;Fink and Meyer, 2002;Rongo, 2002;Elgersma et al., 2004).

The first transgenic mouse generated with altered CaMKII function was the $\alpha CaMKII^{T286A}$ mouse. In this transgenic, the replacement of Thr286 with an alanine (T286A) blocks the ability of the α-subunit to autophosphorylate at this residue which in turn blocks the ability of the kinase to exhibit Ca^{2+}-independent (autonomous)

activity; its Ca^{2+}-dependent catalytic activity, on the other hand, remained unaffected (Giese et al., 1998). This mutation resulted in severe impairments in both CA1 LTP and hippocampal-dependent learning (Giese et al., 1998;Need and Giese, 2003;Irvine et al., 2005). This was a highly significant finding as it was able to link the specific loss of Thr286 autophosphorylation, a property that had been previously hypothesised to underlie the kinase's role in synaptic plasticity (Lisman, 1989;Lisman, 1994), to the resulting LTP and spatial memory impairments exhibited by these mice.

Another advantageous aspect of the $\alpha CaMKII^{T286A}$ mutants over the null mutants is that the transgenic α-subunits will still be incorporated into all heteromeric α/β holoenzymes (the predominant form of CaMKII in the brain) and will thus impair the function of the entire holoenzyme. This is in contrast with the αCaMKII knockout mice where CaMKII holoenzymes still capable of exhibiting high levels autonomous kinase activity will persist in the form of βCaMKII homomers. Furthermore, the cellular functions that do not require Thr286 autophosphorylation but are primarily regulated by Ca^{2+}/CaM-dependent αCaMKII activity will remain unaffected by the point mutation in the $\alpha CaMKII^{T286A}$ transgenics. Thus all cellular and behavioural phenotypes can be directly related to the specific loss of Thr286 autophosphorylation.

The memory deficits exhibited by the $\alpha CaMKII^{T286A}$ transgenics have also been shown to correlate with the instability and decreased selectivity of hippocampal 'place cells' compared to wild-type littermates (Cho et al., 1998). This supports the hypothesis that LTP-like phenomena are required for tuning the firing responses of these cells in response to spatial stimuli in order for the animal to process and learn new spatial information (Cho et al., 1998). NMDA receptor-dependent LTP is also blocked in the neocortex of these mice (Hardingham et al., 2003), as well as experience-dependent synaptic plasticity in both the visual and somatosensory cortices (Glazewski et al., 2000;Glazewski et al., 2001;Taha et al., 2002;Hardingham et al., 2003), supporting the crucial role of Thr286 autophosphorylation in cortical as well as hippocampal synaptic plasticity.

Importantly, neither the voltage dependence of the NMDA receptor nor the average amplitude of evoked NMDA receptor mediated currents were found to be affected by

the T286A mutation; thus the impaired LTP could not be attributed to abnormal NMDA receptor function (Giese et al., 1998). Moreover, several different LTP-inducing stimulation protocols were tested both in the hippocampus and cortex (Giese et al., 1998;Hardingham et al., 2003), again supporting the proposal that the mutation specifically blocks the expression of LTP independent of potentially different induction mechanisms.

If, as hypothesised, the CaMKII-dependent processes that underlie hippocampal-dependent memory formation do involve the specific and long-lasting enhancement of synaptic strengths, it should be possible to detect evidence of such processes having taken place by comparing the characteristics of synaptic efficacy and structure assessed in a normal wild-type brain with those from the brain of $CaMKII^{T286A}$ mutant mice. The detection of any differences would aid the characterisation of endogenous forms synaptic plasticity in the hippocampus and thus of hippocampal learning mechanisms. As described in the first section of this introduction, this forms the overall aim of this Ph.D. thesis.

Giese et al. (1998) also found that the relationship between the amplitude of evoked fibre-volleys (a measure of pre-synaptic activation) and excitatory field potentials (a measure of postsynaptic depolarisation) was indistinguishable between wild-type and $CaMKII^{T286A}$ mutant hippocampi. This indicated a) that overall levels of synaptic connectivity in the CA1 region were not affected by the loss of CaMKII-dependent plasticity and b) that the loss of LTP in the mutants could not be attributed to the pre-potentiation (i.e. saturation) of synaptic strengths prior to induction. Although these measurements do indicate that the summed responses evoked from a large number of inputs are not altered in the $\alpha CaMKII^{T286A}$ mutant mice, it gives no information about possible differences that may exist between the properties of individual wild-type and mutant synapses. Indeed neural network models propose that memory is most likely to exist as a sparse distribution of modified synapses (Willshaw and Dayan, 1990) rather than result from changes involving large numbers of synapses between multiple cells. Furthermore, the net magnitude of excitatory input that impinges onto a CA1 cell would not necessarily be expected to be different between wild-type and LTP-null animals as studies have also suggested that an important aspect of neuronal plasticity involves the

ability to control and stabilise the overall level of excitatory input that a cell receives (Turrigiano and Nelson, 2000). Thus experience-driven synaptic potentiation of some wild-type CA1 inputs might be counteracted by the depression in others or changes in the number of excitatory inputs in order for overall levels of excitatory input to remain constant. For example, experience-dependent plasticity in the wild-type hippocampus might result in the refinement of synaptic connectivity such that any one cell receives fewer inputs that are individually stronger, whereas the lack of such plasticity in the αCaMKII^{T286A} mutant hippocampus may mean that a neurone maintains more excitatory inputs which are individually weaker. Thus, in spite of such differences the overall level of excitatory input to any one neurone may remain comparable between genotypes. On the other hand there may be no changes in synapse number, only a difference in the range and/or distribution of synaptic weights. The experiments reported in this thesis aimed to decipher whether such differences may exist in order to understand the possible consequences of CaMKII-dependent learning and memory in the hippocampus.

1.10 The Molecular Basis of CaMKII Function in Neurones

As discussed earlier, CaMKII is activated by rises in cytosolic Ca^{2+} and its activity is required for the induction of LTP at Schaffer collateral to CA1 synapses, where at least some of its actions result in enhanced AMPA receptor-mediated synaptic transmission. In this section some of the key properties and regulatory features of CaMKII are discussed in order to describe how the loss of Thr286 autophosphorylation is thought to lead to the impairment of LTP in the αCaMKII^{T286A} transgenic mice. As the specific loss of Thr286-dependent CaMKII actions also impairs the endogenous mechanisms that underlie learning and memory in the behaving mouse, the knowledge gained from studies that strive to understand the molecular basis of CaMKII function have also provided important insights into the key processes that may be recruited during learning and memory formation in the brain. An understanding of the known Thr286 autophosphorylation-mediated processes and molecular consequences will also aid the interpretation of any specific differences found between the αCaMKII^{T286A} mutant and wild-type mice that are uncovered in the experiments reported in this thesis. Indeed, although the aim of using the αCaMKII^{T286A} transgenic mice was to investigate the neural consequences resulting from the loss of αCaMKII-dependent synaptic plasticity

in vivo, what the data will really reveal are the effects that result from the loss of all Thr286 autophosphorylation-dependent neural processes. It is known that CaMKII can phosphorylate and/or bind to dozens of target molecules within neurones, including various receptor and channel proteins, scaffolding proteins, cytoskeletal and associated proteins, motor proteins, other enzymes and proteins involved in neurotransmitter release (Braun and Schulman, 1995;Fink and Meyer, 2002). Although the broad spectrum of targets renders CaMKII ideally suited to a role in translating changes in Ca^{2+} into cell function, the fact that the functions of many of these interactions remain unknown means that the loss of CaMKII Thr286 autophosphorylation in the mutant mice may also affect cellular processes other than those recognised as contributing to increased synaptic efficacy. Thus a good understanding of CaMKII biochemistry and physiology is important in order to attempt to appreciate some of the other consequences that might result, either directly or indirectly, from the loss of Thr286 autophosphorylation. These issues however will primarily be discussed in the discussion section of this thesis, in light of the differences reported within the results section.

1.10.1 CaMKII Isoforms and Expression Patterns

There are four distinct CaMKII isoforms (α, β, γ and δ) expressed in mammals that are derived from four closely related genes. Each of the four CaMKII gene products are subject to alternative splicing which gives rise to multiple species of each isoform and to date more than 30 distinct gene products have been identified (Hudmon and Schulman, 2002).

The CaMKII isoforms are differentially expressed through mammalian tissues. The α- and β-isoforms are specific to and are the predominant isoforms found in brain, whereas the γ- and δ-isoforms show more ubiquitous expression patterns throughout the body (Tobimatsu and Fujisawa, 1989;Bayer et al., 1999). Using Northern blot analysis, Bayer et al. (1999) have shown that mRNA for the β-, γ- and δ-isoforms are all expressed during embryonic development, but they were unable to detect a signal for the α-isoform in mouse brain until postnatal day 5 (P5). In one day old mice, a weak signal for the α-isoform could, however, be detected within the CA2 region of the

hippocampus using *in situ* hybridisation techniques in brain slices (Bayer et al., 1999).

The expression of predominant α- and β-isoforms in the brain is not ubiquitous, with the highest expression levels being found in the hippocampus and frontal cortex (Ouimet et al., 1984;Erondu and Kennedy, 1985;Burgin et al., 1990)). Furthermore, the α-isoform is almost exclusively found in excitatory neurones in the brain, although the βCaMKII has been found in both excitatory and inhibitory neurones (Ochiishi et al., 1994;Sik et al., 1998;Thiagarajan et al., 2002) as well as in gial cells (Ouimet et al., 1984).

The relative expression levels of the α- and β-isoforms also differ in both a regional- and developmental stage-specific manner (Burgin et al., 1990). This has particular functional significances because the relative expression levels of the two isoforms dictates the ratio of the isoforms within a kinase holoenzyme (Brocke et al., 1999), which in turn influences the functional properties of the kinase as a whole (De Koninck and Schulman, 1998; see section 1.10.8). Furthermore, as the developmental profiles of the isoforms within a brain region are also different this means that the functional properties of the holoenzyme are also likely to change with age. This may underlie certain developmental-stage specific functions of the CaMKII holoenzyme in neurones (e.g. Fink et al., 2003).

In the forebrain, the levels of αCaMKII mRNA increase between postnatal day 4 (P4) and adulthood; approximately 10-fold in frontal cortex and 7-fold in hippocampus (Burgin et al., 1990). These changes were seen to occur mostly within the second postnatal week (Burgin et al., 1990) and agrees with the observed age-dependent increases in αCaMKII protein levels seen in synaptic-junction subcellular fractions of rodent forebrain (Kelly and Vernon, 1985;Kelly et al., 1987). In the cerebellum, αCaMKII mRNA is at approximately 50% of adult levels at P4 and increases during the third postnatal week to adult intensities by P18 (Burgin et al., 1990). In contrast, the total brain levels of βCaMKII are already high at P4 and actually decrease slightly during the first weeks of postnatal life, reaching adult-like brain intensities by 3 weeks of age (Burgin et al., 1990). This decrease is primarily due to the reductions found in the

cortex, where levels half between P4 and adulthood.

In the hippocampus, the study by Burgin et al. found that levels of βCaMKII were already adult-like at P4 and remained stable throughout the first postnatal month and until at least 3.7 months of age (the oldest animals tested). This is a large contrast to the 5-fold increase seen between P4 and P16 for the α-isoform in the hippocampus; no further increases were observed thereafter through to adulthood. In the cerebellum, βCaMKII intensities were already 70% of adult levels by P4 and increases were found to occur over the same time-scale as the α-isoform, also reaching adult levels by P18. Coincidently, these increases in CaMKII coincide with the main period of neuro- and synaptogenesis in the cerebellum (Burgin et al., 1990;Armengol and Sotelo, 1991).

The parallel increases of each isoform through cerebellar development mean that the ratio of α- to β-isoforms (1:4) and thus the properties of the holoenzyme do not change with time (McGuinness et al., 1985;Burgin et al., 1990). In contrast, the distinct developmental profiles of these isoforms in the rat forebrain translate into drastic changes of the α:β ratio during development. During embryonic stages, the enzyme is likely to be primarily composed of βCaMKII since the α-subunit has only been detected postnatally (Bayer et al., 1999). At P4 and P5, the ratio sits at 1:7 (Kelly et al., 1987;Burgin et al., 1990), increasing to 1:3 by P10 (Rostas et al., 1988) and reaching 3:1 by adulthood (McGuinness et al., 1985;Kelly et al., 1987). The physiological significance of the potential changes in CaMKII function through development has not yet been investigated directly. One potential consequence could be that the altering holoenzyme composition enables the kinase to optimally tune its responses to Ca^{2+} signals that change through development; this could be achieved because of the distinct affinities of the α- and β-subunits for Ca^{2+}/CaM (De Koninck and Schulman, 1998; see section 1.10.8). Another important difference between the α- and β-isoforms is that only the β-isoform contains an insert that enables the subunit to bind to filamentous actin (F-actin; Shen et al., 1998). Thus the higher levels of βCaMKII in early development would also ensure that the kinase to targeted to the actin cytoskeleton of neurones, whereas homomeric αCaMKII holoenzymes that may also occur in adult neurones would not be targeted in this way (Shen et al., 1998). Indeed, a specific role has been suggested for βCaMKII in neonatal neurones of the hippocampus in relation to the

regulation of neurite extensions, the branching of filopodia and fine dendrites, and the control of synapse number (Fink et al., 2003).

Differences between the α- and β-isoforms also exist with respects to their subcellular distributions in hippocampal and cortical pyramidal neurones. While protein for both isoforms can be detected within the cytoplasm of the perikarya and dendrites and accumulate at PSDs (where CaMKII forms 2-3% of PSD protein (Erondu and Kennedy, 1985)) only the α-isoform is also targeted to the nuclei of pyramidal cells (Ochiishi et al., 1994). Indeed, CaMKII activity in the nucleus has been shown to lead to the regulation of various transcription factors, including cyclic-AMP response binding protein (CREB) (Matthews et al., 1994;Sun et al., 1996;Blanquet et al., 2003) which is an important transcription factor required for long-term synaptic plasticity (Mower et al., 2002;Nguyen and Woo, 2003) and memory formation (Yin and Tully, 1996;Tully et al., 2003). Note, within the hippocampus, the specific staining patterns for the α and βCaMKII isoforms are similar for each of the distinct subfields (i.e. CA1, CA2 and CA3; Ochiishi et al., 1994).

The presence of αCaMKII protein in pre-synaptic nerve terminals has also been demonstrated for neurones of the cerebral cortex, striatum and hippocampus (Gorelick et al., 1988;Walaas et al., 1989;Liu and Jones, 1996). In the cortex, the staining of αCaMKII in nerve terminals was found to be specific for glutamatergic synapses, although not all glutamatergic terminals were labelled, and αCaMKII was never found to be associated with GABAergic synapses, neither pre- nor post-synaptically (Liu and Jones, 1996). This would suggest that in the cerebral cortex αCaMKII does not play a role in the plasticity of inhibitory synapses.

As described above, protein for both isoforms is located throughout the dendritic processes of pyramidal cells in the hippocampus (Ochiishi et al. 1994). However, differences exist between the two isoforms in relation to the targeting of their mRNA transcripts. The βCaMKII transcript is restricted to the soma of both pyramidal and granule cells, whereas mRNA for αCaMKII is also distributed throughout the dendrites, resulting in diffuse labelling throughout the 3 distinct hippocampal laminae (*stratum oriens*, *stratum radiatum* and *stratum lacunosum moleculare*) and the *stratum*

moleculare of the dentate gyrus (Burgin et al. 1990). These data suggest that dendritic translation of αCaMKII mRNA into protein may therefore be important for αCaMKII function in synaptic plasticity. Indeed, tetanic stimulation has been found to lead to a rapid increase in αCaMKII protein levels in the stratum radiatum of CA1 (Ouyang et al., 1997), which could be blocked by mRNA translation inhibitors (Ouyang et al., 1999). Moreover, the importance of dendritic translation of αCaMKII in synaptic plasticity and memory was recently demonstrated directly by the development of a transgenic mouse where the identified dendritic localization signal within the 3' untranslated region of the mRNA was mutated (Miller et al., 2002). The mutation caused a dramatic reduction in αCaMKII protein levels at the PSD and although the early and intermediate phases of LTP were intact, a significant reduction in late-phase LTP was observed (assessed 4 hours after tetanus stimulation). The animals also showed severe impairments in the Morris water maze test for spatial reference memory as well as in long-term memory tests of cued and contextual conditioning and object recognition memory (tested 24 hours after training). Interestingly short term memory of these latter tests were not impaired (tested 30 minutes and 1 hour after training respectively) correlating with intact LTP at these time points.

1.10.2 CaMKII structure and holoenzyme assembly

Each CaMKII isoform consists of several conserved domains: a highly conserved N-terminal catalytic domain (~ 280 amino acids), followed by an adjacent regulatory domain (~ 40 amino acids), a linker domain and a C-terminal association domain (150-220 amino acids; figure 1.4 A). The regulatory domain contains the autoinhibitory domain, a CaM-binding domain and several regulatory autophosphorylation sites that are autophosphorylated via intra-holoenzyme reactions. These sites correspond to residues Thr286, Thr305 and Thr306 within the αCaMKII isoform, and residues Thr287, Thr306 and Thr307 within the β-, γ- and δ-isoforms. The catalytic domain contains the ATP and substrate binding sites and the functional ability to catalyse the phospho-transferase reaction with target substrates. The catalytic domain also contains sites for interaction with anchoring proteins. Finally, the catalytic domain is able to associate with the autoinhibitory domain, a critical process for CaMKII self-inhibition in the basal state. The C-terminal association domain allows the assembly of a non-

Figure 1.4 Schematics of CaMKII Structure and Assembly.

A) A linear schematic of an αCaMKII subunit indicating the functional domains of the primary structure. Within the regulatory domain, the amino acids that make up the autoinhibitory and calmodulin binding regions are shown. The specific threonine (T) residues that form autophosphorylation sites are indicted.

B) Schematic representations of the domain organisation of:

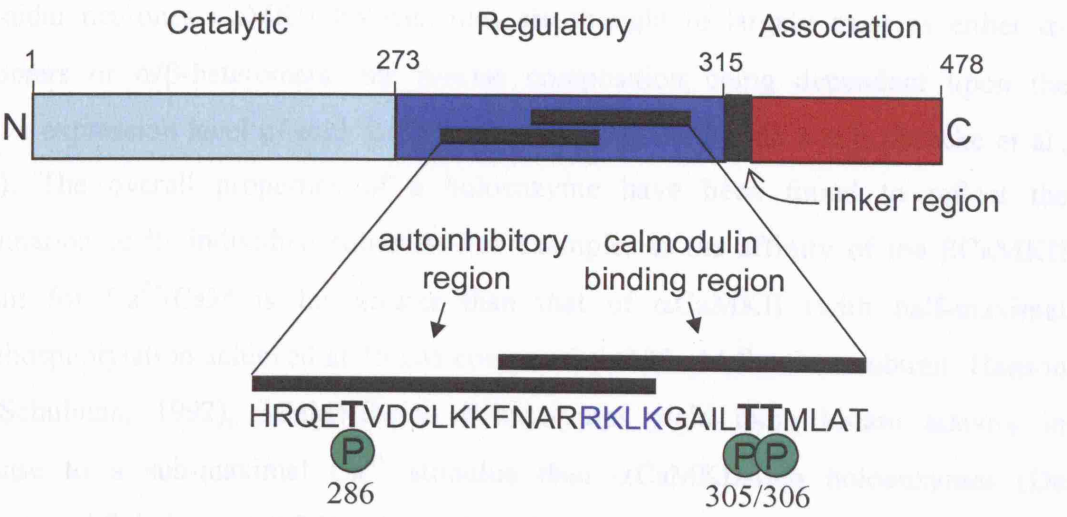
i) A single CaMKII subunit.

and

ii) The double hexamer holoenzyme.

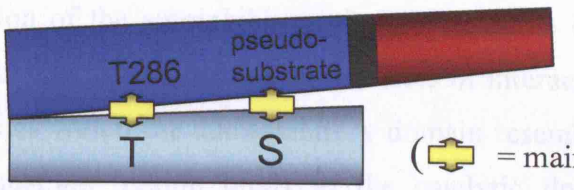
All subunits are shown in the inactive (closed-gate) configuration. The inactive state of a CaMKII subunit is due to the interactions formed between the catalytic and regulatory domain; the pseudosubstrate region within the autoinhibitory domain interacts with the catalytic domain within the ATP / substrate-binding (S) site, and the autophosphorylation site Thr286 interacts with a so-called T-site within the catalytic domain.

A)



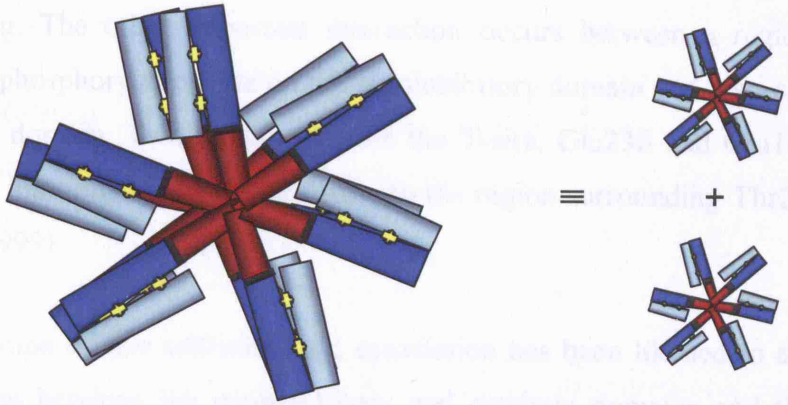
B)

i)



(= main sites of interaction)

ii)



dissociable holoenzyme. The functional enzyme exists as a holoenzyme made up of twelve individual CaMKII subunits that associate via their C-terminal domains (figure 1.4 B). The subunits arrange themselves into a stack of two hexameric rings (known details of holoenzyme structure are reviewed in Hudmon and Schulman, 2002). In CA1 pyramidal neurones CaMKII holoenzymes are thought to largely exist as either α-homomers or α/β-heteromers; the precise composition being dependent upon the relative expression level of each isoform at any given site within a cell (Brocke et al., 1999). The overall properties of a holoenzyme have been found to reflect the combination of its individual subunits. For example, as the affinity of the βCaMKII subunit for Ca^{2+}/CaM is far greater than that of αCaMKII (with half-maximal autophosphorylation achieved at 15 nM compared to 130 nM for the -subunit; Hanson and Schulman, 1992), βCaMKII-rich holoenzymes yield more kinase activity in response to a sub-maximal Ca^{2+} stimulus than αCaMKII-rich holoenzymes (De Koninck and Schulman, 1998;Brocke et al., 1999).

1.10.3 Autoinhibition in the Basal State

In the absence of Ca^{2+}/CaM, CaMKII remains inactive in a self-inhibited state. This is due to the interaction of the autoinhibitory domain with the catalytic domain within each holoenzyme subunit. There are two known sites of interaction (figure 1.4 B). One of the interacting sites within the autoinhibitory domain resembles a protein substrate, and this 'pseudosubstrate' region binds to the catalytic domain at the ATP and substrate-binding regions (termed the S-site; Colbran et al., 1989;Smith et al., 1992;Mukherji and Soderling, 1995), thus preventing ATP and any genuine substrate from binding. The other important interaction occurs between a region around the Thr286 autophosphorylation site on the autoinhibitory domain and a so-called T-site on the catalytic domain. Two residues within the T-site, Glu236 and Glu109, have been identified as important for the association to the region surrounding Thr286 (Yang and Schulman, 1999).

The organisation of this self-inhibiting association has been likened to a 'gate' with a hinge existing between the autoinhibitory and catalytic domains and the gate being closed in the basal state (Lisman et al., 2002). The Thr286 binding region and opposing

T-site are situated at either side of the 'hinge' of this gate-like structure, and their association is required for the correct positioning and thus interaction of the pseudosubstrate binding site and S-site (Yang and Schulman 1999 (Yang and Schulman, 1999). Association of the Thr286 binding region with the T-site is also thought to induce secondary conformational changes that interfere with ATP molecules binding to the catalytic domain; this itself preventing kinase activity (Lengyel et al. 2001 (Lengyel et al., 2001b).

1.10.4 Ca^{2+}/CaM-Dependent Activation and Autophosphorylation

CaMKII is activated by the binding of Ca^{2+}/CaM to a region within its autoinhibitory domain (residues 293-310) that overlaps with the pseudosubstrate region. The binding of Ca^{2+}/CaM is therefore thought to disrupt the interaction between the autoinhibitory and catalytic domains and cause the conformational "opening-up" of the CaMKII subunit, allowing both ATP and substrates access to the catalytic site (Smith et al. 1992 (Smith et al., 1992). Indeed, certain amino acids have been identified in the CaMKII regulatory domain that are critical for both Ca^{2+}/CaM binding and for its association with the S-site, supporting this theory (Yang and Schulman, 1999). Importantly, the binding of Ca^{2+}/CaM also results in the exposure of Thr286 which enables the subunit to be autophosphorylated by a neighbouring activated subunit within the holoenzyme (Hanson et al., 1989). Thus, for Thr286 autophosphorylation to occur, the coincident binding of two Ca^{2+}/CaM molecules must take place, one to the subunit possessing the exposed Thr286 substrate and one to the adjacent subunit which can in turn phosphorylate its neighbour at residue Thr286 (reviews include: Hudmon and Schulman, 2002;Colbran, 2004;Griffith, 2004). Once one subunit is autophosphorylated and the binding of only one further Ca^{2+}/CaM molecule is required to initiate further autophosphorylation which can ultimately propagate around the entire ring. This cooperative nature of Thr286 autophosphorylation and its proposed role in enabling CaMKII to act as a frequency detector of Ca^{2+} oscillations is discussed further in section 1.10.8.

Breaking the interaction between the catalytic and autoinhibitory domains also enables CaMKII to associate with other proteins within a cell; for example, permitting CaMKII to bind to proteins within the PSD, thus anchoring the CaMKII to the postsynaptic

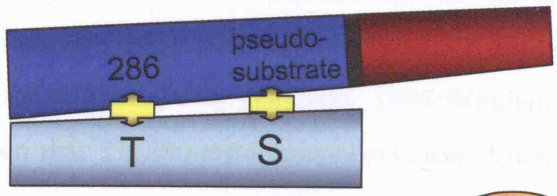
membrane. Several proposed binding sites have been identified within the catalytic domain of CaMKII, including a site that is required for the binding of CaMKII to the NMDA receptor (Bayer et al., 2001).

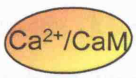
Thr286 autophosphorylation has two important consequences for kinase function. First, it enables Ca^{2+}/CaM-independent catalytic activity (Miller et al., 1988;Thiel et al., 1988). This is achieved because autophosphorylation at Thr286 disables the autoinhibitory domain by sterically preventing the gate from closing; the positioned phosphate has been likened to the placing a block near the hinge of an open gate preventing it from closing (Lisman et al., 2002; see figure 1.5). With Thr286 autophosphorylated and the surrounding amino acids no longer associated to the T-site of the catalytic domain, the substrate-binding catalytic S-site is therefore able to remain exposed and active, even when Ca^{2+} levels fall and Ca^{2+}/CaM dissociates away from the autoinhibitory domain (Miller and Kennedy, 1986). This is referred to as "autonomous" or Ca^{2+}/CaM-independent activity and it will persist until Thr286 is dephosphorylated by phosphatase activity. This ability of CaMKII to remain active even after Ca^{2+} levels have returned to basal levels was immediately recognised as having the potential to play an important role in synaptic plasticity by being able to hold a biochemical memory of past supra-threshold levels of synaptic activity (Miller and Kennedy, 1986). This also agrees with previous theoretical models of memory storage that predicted that such an enzyme would exist (Lisman, 1985). It was initially thought that autonomous activity would play a crucial role in prolonging kinase activity by being able to oppose the de-phosphorylating actions of neuronal phosphatases and maintaining a highly autophosphorylated state of the CaMKII holoenzyme (Lisman, 1985;Miller and Kennedy, 1986;Lisman and Goldring, 1988); however it is now recognised that the maintenance of Thr286 autophosphorylation cannot be dependent upon only autonomous activity as it requires that Ca^{2+}/CaM is bound in order to expose the Thr286 residue (Hanson et al., 1994), thus the maintenance of a highly Thr286 autophosphorylated is also Ca^{2+}-dependent (Lisman and Zhabotinsky, 2001). Note, the level of catalytic activity produced by an autonomous kinase is only 20-39% of the maximum that is attained when Ca^{2+}/CaM is still bound to CaMKII (Miller and Kennedy, 1986;Fong et al., 1989;Meyer et al., 1992).

Figure 1.5 Ca^{2+}/CaM-Dependent Activation and Autophosphorylation.

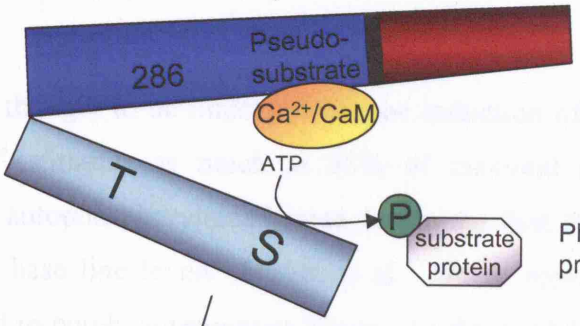
This figure summarises the different activity states of a single CaMKII subunit. An inactive CaMKII subunit (**1**) is relieved from autoinhibition by the association of a molecule of Ca^{2+}/CaM (**2**). The binding of Ca^{2+}/CaM results in the exposure of the ATP / substrate-binding region within the catalytic domain, rendering the subunit catalytically active and able to phosphorylate target substrates. If an adjacent CaMKII subunit within the holoenzyme also becomes active before Ca^{2+}/CaM has dissociated from the active subunit, intra-subunit autophosphorylation is able to occur at residue Thr286 (**3**). This allows the subunit to remain in the 'open' and active configuration even when Ca^{2+}/CaM has dissociated; Thr286 autophosphorylation-dependent kinase activity in the absence of Ca^{2+}/CaM is referred to as 'autonomous' activity. Thr286 autophosphorylation also results in a large increase in the affinity of CaMKII for bound Ca^{2+}/CaM and is termed CaM-trapping (**3**). Once Ca^{2+}/CaM has dissociated, 'burst' or 'inhibitory' autophosphorylation takes place at residues Thr305 and Thr306 (**4**). Until these sites are dephosphorylated by phosphatase action, CaMKII is unable to rebind Ca^{2+}/CaM.

1) An inactive CaMKII subunit
in the 'closed-gate' configuration.

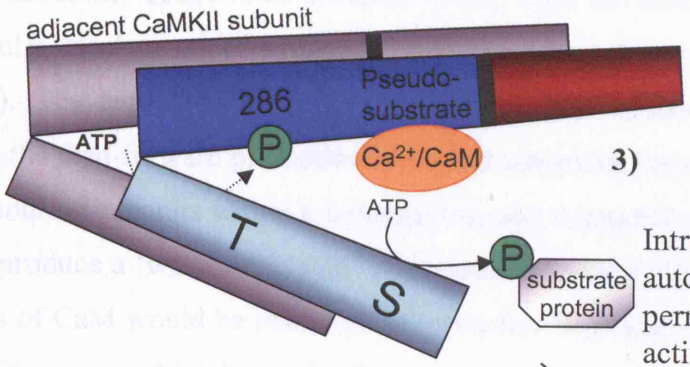




2) Ca^{2+}/CaM-dependent
activation and kinase
activity

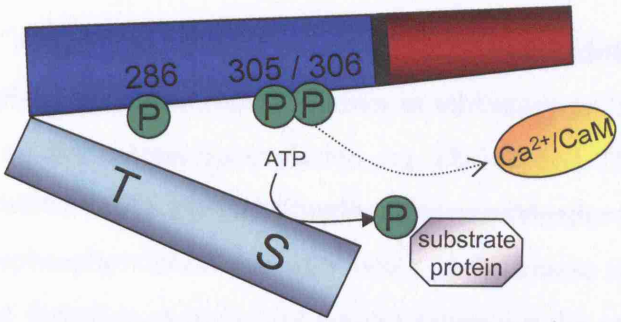


Phosphorylation of
protein substrates



3)

Intra-holoenzyme Thr286
autophosphorylation
permits autonomous
activity and CaM-trapping



4) CaM dissociation
initiates 'inhibitory'
autophosphorylation
at Thr305and Thr306

The second consequence of Thr286 autophosphorylation is a 1000-fold increase in the affinity of the enzyme for CaM, producing the phenomenon known as 'CaM-trapping' (Meyer et al., 1992). It is thought that Thr286 autophosphorylation induces a local conformation change in the CaMKII peptide that allows the formation of additional stabilizing interactions between CaM and the regulatory domain of CaMKII that enables this CaM-trapped state (Putkey and Waxham, 1996;Waxham et al., 1998;Singla et al., 2001). It was shown that Thr286-autophosphorylation slows the release time of bound CaM from less than a second to several hundred seconds (Meyer et al., 1992). Indeed, Thr286 autophosphorylated CaMKII exhibits the highest known binding affinity for CaM, whereas the unphosphorylated form exhibits one of the lowest (Meyer et al., 1992).

CaM-trapping is thought to be important for the induction of synaptic plasticity, since following a Ca^{2+} stimulus as much as 85% of maximal kinase activity has been measured for the autophosphorylated kinase during the first 10 seconds that Ca^{2+} levels have returned to base line levels (Meyer et al., 1992); more than twice the level of activity compared to purely autonomous kinase. As the availability of CaM is limited in a cell, the ability of activated synapses to trap CaM has also been proposed to provide a molecular mechanism of synaptic competition in neurones (Okamoto and Ichikawa, 2000a). The autophosphorylation of CaMKII and subsequent CaM-trapping would enable the feed-forward promotion of further autophosphorylation and CaM-trapping on neighbouring subunits within a holoenzyme, and computer modeling has found that this could produce a 'winner-takes-all' phenomenon to take place in neurones such that the actions of CaM would be restricted to just a few activated synapses at any one point in time (Okamoto and Ichikawa, 2000a).

Following the dissociation of Ca^{2+}/CaM from Thr286 autophosphorylated CaMKII, a second distinct phase of autophosphorylation it initiated, known as inhibitory or 'burst' autophosphorylation. The targets of this autophosphorylation are Thr305 and Thr306 (Thr307 for βCaMKII subunits) situated within the CaM-binding domain (Meador et al., 1993). Importantly, inhibitory autophosphorylation can only occur if the kinase is still autophosphorylated at Thr286 (and therefore catalytically active) following the release of CaM. The presence of phosphate groups at either Thr305 or Thr306 prevents the

rebinding of Ca^{2+}/CaM and, therefore, maximal kinase activity (i.e. Ca^{2+}/CaM-dependent activity); although while Thr286 also remains phosphorylated, autonomous activity can continue (Patton et al., 1990;Hanson and Schulman, 1992). CaM-trapping also has the important function, therefore, of masking these inhibitory phosphorylation sites and thus delaying the formation of the Thr305/306-autophosphorylated state. The re-binding of Ca^{2+}/CaM is therefore subject to a phosphatase-dependent refractory period (Meyer et al., 1992).

Slow basal phosphorylation of Thr306 has also been observed in the absence of any measurable levels of CaMKII activity, producing a kinase that cannot therefore be activated by Ca^{2+}/CaM (Hanson and Schulman, 1992;Colbran, 1993). This observation could be related to the findings from a recent study in drosophila that studied the consequences of CaMKII association to Camguk (a specific membrane-associated guanylate kinase). They found that in the absence of Ca^{2+}/CaM, the binding of CaMKII to Camguk selectively stimulated inhibitory autophosphorylation at residues Thr305 and Thr306 (Lu et al., 2003a). On the other hand, in the presence of Ca^{2+}/CaM, CaMKII complexed with Camguk could autophosphorylate at Thr286 and thus become constitutively active (Lu et al., 2003a). The authors suggest that the binding of CaMKII to Camguk may enable neurones to regulate the level of CaMKII activity on a local level and thus differentiate between recently active and inactive synapses. Specifically, the association of CaMKII to Camguk during prolonged periods of inactivity and low levels of intracellular Ca^{2+} would promote and act to down-regulate the Ca^{2+}-stimulatable pool of CaMKII through inhibitory phosphorylation. In these situations, phosphatase activity would be required to reverse the down-regulated CaMKII levels of activity and stimulate increased CaMKII activity.

1.10.5 The Dephosphorylation of CaMKII by Protein Phosphatases

There are two main protein phosphatases responsible for dephosphorylating neuronal CaMKII (i.e. at its autophosphorylation sites): PP1 and PP2A (Shields et al., 1985). PP2C is also capable of dephosphorylating CaMKII although its contribution (at least under *in vitro* conditions) is very small and due to the lack of specific inhibitors nothing is known about its possible roles *in situ* (Shields et al., 1985;Fukunaga et al., 1993a;Strack et al., 1997a). PP2B does not exhibit activity towards the CaMKII

autophosphorylation sites.

CaMKII is differentially regulated by PP1 and PP2A depending on its subcellular location. When CaMKII is bound to the PSD, only PP1 is able to dephosphorylate the kinase and this is thought to be catalysed through the ability of the γ1-isoform of PP1 to anchor to the PSD via specific scaffolding proteins (Strack et al., 1997a;Yoshimura et al., 1999). As discussed earlier, the activity-dependent control of PP1 activity levels via the PP2B/PKA-dependent regulation of its endogenous inhibitor protein, inhibitior-1, plays a crucial role in the synaptic plasticity.

PP2A, on the other hand, is somehow excluded from the PSD but this phosphatase is important for the dephosphorylation of non-synaptic CaMKII (Strack et al., 1997a). Despite its absence from the synapse, the control of PP2A activity has also been proposed to play a role in synaptic plasticity. LTP in the CA1 region of the hippocampus was found to be associated with a significant decrease in PP2A activity that appeared to result from the direct phosphorylation of its regulatory subunit by CaMKII (Fukunaga et al., 2000). Thus, although the negative regulation of PP2A is probably not involved in the induction of LTP at the synapse, it might be important for the expression of LTP by maintaining the phosphorylation states of PP2A substrates outside of the PSD.

Another protein phosphatase has been identified more recently that specifically dephosphorylates all of the multifunctional Ca^{2+}/CaM-dependent protein kinases and was thus termed CaMK phosphatase (CaMKP) (Ishida et al., 1998). CaMKP exists as a monomer that is ubiquitously distributed and it is activated by CaMKII-dependent phosphorylation. Since the autophosphorylated form of CaMKII is also a substrate of CaMKP is has been proposed that CaMKP activation by CaMKII may offer a kind of negative feedback regulation (Kameshita et al., 1999), although a specific role of CaMKP in synaptic physiology remains to be uncovered. Nevertheless, one study has shown that although the content of CaMKP in the PSD is usually very low, PSDs clearly stained for CaMKP using specific antibodies could occasionally be seen in some neurones (Nakamura et al., 2000). It was therefore suggested that CaMKP may participate in the dephosphorylation of synaptic CaMKII, but only under some

conditions (Ishida et al., 2003).

1.10.5.1 The Regulation of PP1 Activity and Synaptic Plasticity

As discussed earlier in section 1.7.2, the activity levels of PP1 are regulated in an activity-dependent manner that depends upon the phosphorylation state of its endogenous inhibitor protein, inhibitor 1; small increases in intracellular Ca^{2+} concentration enhance PP1 activity via the PP2B-dependent dephosphorylation of inhibitor 1, preventing its association to, and inhibition, of PP1 while larger increases in intracellular Ca^{2+} concentration reduce PP1 activity via the PKA-mediated phosphorylation of inhibitor 1. This activity-dependent regulation of PP1 is widely considered to play an important role in the bidirectional control of synaptic strength, learning and memory (Mansuy, 2003;Ishida et al., 2003;Munton et al., 2004). It has even been proposed that a specific role of PP1 activity may be to provide a negative constraint upon memory formation and that the activity-dependent up-regulation of PP1 inhibition therefore provides a mechanism by which the brain can select between events that are more meaningful over those less significant that do not impair PP1 activities (Silva and Josselyn, 2002); i.e. the enhanced inhibition of PP1 permits more significant events to be encoded into long-term memories via the permissive gating of LTP whilst those less important for survival are discarded via phosphatase activity (Blitzer et al., 1998;Genoux et al., 2002).

It might, therefore, be expected that learning and memory would be impaired in a transgenic mouse lacking the inhibitor-1 gene; however, spatial learning in the Morris water maze as well as LTP at Schaffer collateral synapses were found to be unaffected in such mutants compared to wild-type control animals (Allen et al., 2000). Thus, the activity-dependent regulation of inhibitor-1 may not be the only way by which PP1 activity can be reduced in order to gate CaMKII activities. Indeed, the activity of PP1 is also known to be influenced by its association with specific scaffolding proteins at the synapse; thus, as suggested by Allen and colleagues (2000), this may also play an important role in the activity-dependent regulation of PP1 and the induction of LTP. Three such scaffolding proteins have received particular attention regarding their potential roles in the targeting of PP1 to the synaptic compartment and the regulation of PP1 activity and synaptic transmission; they are yotiao, spinophilin and neurabin

(reviewed in Winder and Sweatt, 2001).

Yotiao binds to the NR1 subunit of the NMDA receptor and is present in the hippocampus, cerebral cortex and cerebellum (Lin et al., 1998). It also binds PP1 and the type-II regulatory subunit of PKA and it has been indicated *in vitro* that the binding of PP1 to yotiao can tonically inhibit the NMDA receptor (Westphal et al., 1999). Rising cAMP levels may then contribute to synaptic potentiation by stimulating the yotiao-bound PKA and favouring the phosphorylation of the NMDA receptor and thus opposing PP1 activity. Thus yotiao may also contribute towards synaptic potentiation by simply placing PP1 in close proximity to PKA and also, therefore, with phosphorylated inhibitor-1. This optimal location of such a kinase-phosphatase signalling complex could contribute to the gating of CaMKII activity, although it has also been suggested that it may play a more prominent role in the control of the phosphorylation state of the NMDA receptor (Westphal et al., 1999).

Spinophilin, on the other hand, could provide an alternative mechanism for the activity-dependent inhibition of PP1. The function of spinophilin has received a great deal of attention because it is highly concentrated in dendritic spines and inhibits the activity of PP1, to which it binds with a high affinity (Allen et al., 1997;Muly et al., 2004). Spinophilin also contains a PDZ binding domain which implicates that it can cross-link bound PP1 to other membrane-associated proteins such as neurotransmitter receptors. Indeed, spinophilin has been shown to bind to a variety of synaptic receptors including D2 dopamine receptors (Smith et al., 1999) and α2-adrenergic receptors (Richman et al., 2001). The role of spinophilin in PP1 inhibition appears to be quite complicated. Although the binding of spinophilin to PP1 reduces phosphatase activity *in vitro* (Allen et al., 1997), it has been suggested that spinophilin may actually act to present PP1 to target synaptic proteins and negatively modulate synaptic transmission (Yan et al., 1999). Yan et al. have proposed that in striatal neurones, the binding of PP1 to spinophilin acts to concentrate PP1 at the PSD and allows PP1 to regulate the phosphorylation state of AMPA receptors – possibly via transient dissociation of PP1 from spinophilin (Yan et al., 1999). They found that competing for the PP1 interaction with spinophilin using a short PP1 peptide reduced the rundown of agonist-evoked

AMPA receptor currents, perhaps by reducing the concentration of PP1 at the synapse.

In contrast, the binding to spinophilin might act to decrease the concentration of PP1 at the PSD in an activity-dependent manner. One study has shown that stimulation of dopamine receptors in striatal neurones *in vitro* resulted in the phosphorylation of spinophilin by PKA which in turn disrupted the association of spinophilin with the actin cytoskeleton (Hsieh-Wilson et al., 2003). After sub-cellular fractionation, they found that while the unphosphorylated spinophilin remained enriched in the PSD, phosphorylated spinophilin was localised in the cytosolic fraction, taking PP1 with it as its ability to bind PP1 remained unchanged. Thus PKA activation may act to decrease the availability of PP1 at the synapse as well as enhancing PP1 inhibition through the phosphorylation of inhibitor 1.

Neurabin (the third main PP1 binding protein), like spinophilin, inhibits PP1 activity, contains a PDZ domain and binds to actin. However, in contrast with spinophilin, *in vitro* studies show that its binding affinity for PP1 is significantly reduced after phosphorylation by PKA (McAvoy et al., 1999). This could also therefore result in a decrease in the availability of synaptic PP1 and decrease phosphatase activity at synaptic sites in an activity-dependent manner.

In conclusion, the targeting of PP1 to protein signalling complexes within dendritic spines via its association with proteins such as spinophilin, neurabin and yotiao, may play important, and probably distinct, roles in the regulation of PP1 and therefore in control of bidirectional synaptic plasticity.

1.10.6 The Activity-Dependent Translocation of CaMKII to the PSD

Under basal conditions, neuronal CaMKII makes up as much as 2-3 % of the constituent protein at the PSD (Erondu and Kennedy, 1985;Suzuki et al., 1994). Although this is much lower than the earlier estimates that showed that CaMKII constituted 20-50% of total protein in isolated PSD preparations (Kennedy et al., 1983;Kelly et al., 1984). Indeed it is now known that these over-estimates were a direct result of decapitation-induced translocation and association of CaMKII to the PSD (Suzuki et al., 1994). This finding corresponded with the then already known phenomenon of depolarisation-

induced thickening of the PSD (e.g. Kotlyar et al., 1990;Yen et al., 1993) and it has now been shown using immuno-electron microscopy that this thickening is due, as least in part, to activity-induced accumulation of CaMKII at the cytoplasmic face of the PSD (Dosemeci et al., 2001). Indeed the 3-dimensional positioning and distribution of CaMKII within the PSD has been described in some detail (Petersen et al., 2003) and the translocation of CaMKII to synaptic sites has also been demonstrated *in vivo* (Gleason et al., 2003). This activity-dependent translocation of CaMKII into synapses is now widely acknowledged as playing an important role in the function of CaMKII in synaptic plasticity (Lisman et al., 2002;Schulman, 2004). Figure 1.6 illustrates the temporal sequence of the events and activity states of CaMKII that bring about the activity-dependent translocation and binding of CaMKII to the PSD as well as those that control its subsequent release.

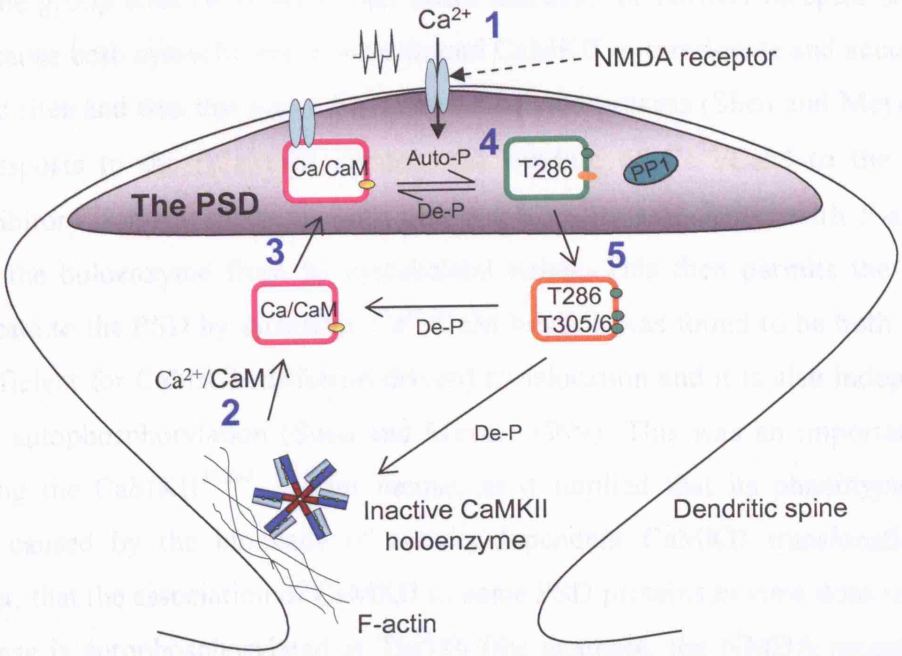
Under resting conditions, the localisation of non-synaptic CaMKII is governed by the subunit composition of the holoenzyme. Evidence for this first came from studying transfected green fluorescent protein (GFP)-tagged α- and βCaMKII subunits in cultured hippocampal neurones (Shen et al., 1998). Shen and colleagues found that βCaMKII subunits would bind to the F-actin in dendritic spines and filopodia and to the cytoskeletal cortex of the cell. In contrast GFP-tagged αCaMKII remained largely cytosolic, uniformly distributed throughout the soma and dendritic processes and largely absent from spines. When expressed together the α and β GFP-tagged isoforms were able to form heteromeric holoenzymes that resulted in the targeting of both isoforms to the actin structures. Their experiments also indicated that only a small number of β-subunits per holoenzyme were required in order to dock the α/β CaMKII hetero-oligomeric holoenzymes to F-actin. Indeed, since forebrain CaMKII is predominantly composed of the α-subunit (approximately 9 - and 3 -subunits per holoenzyme; McGuinness et al., 1985;Miller and Kennedy, 1985) a specific role of the βCaMKII under baseline conditions is likely to be the targeting of CaMKII to the actin cytoskeleton.

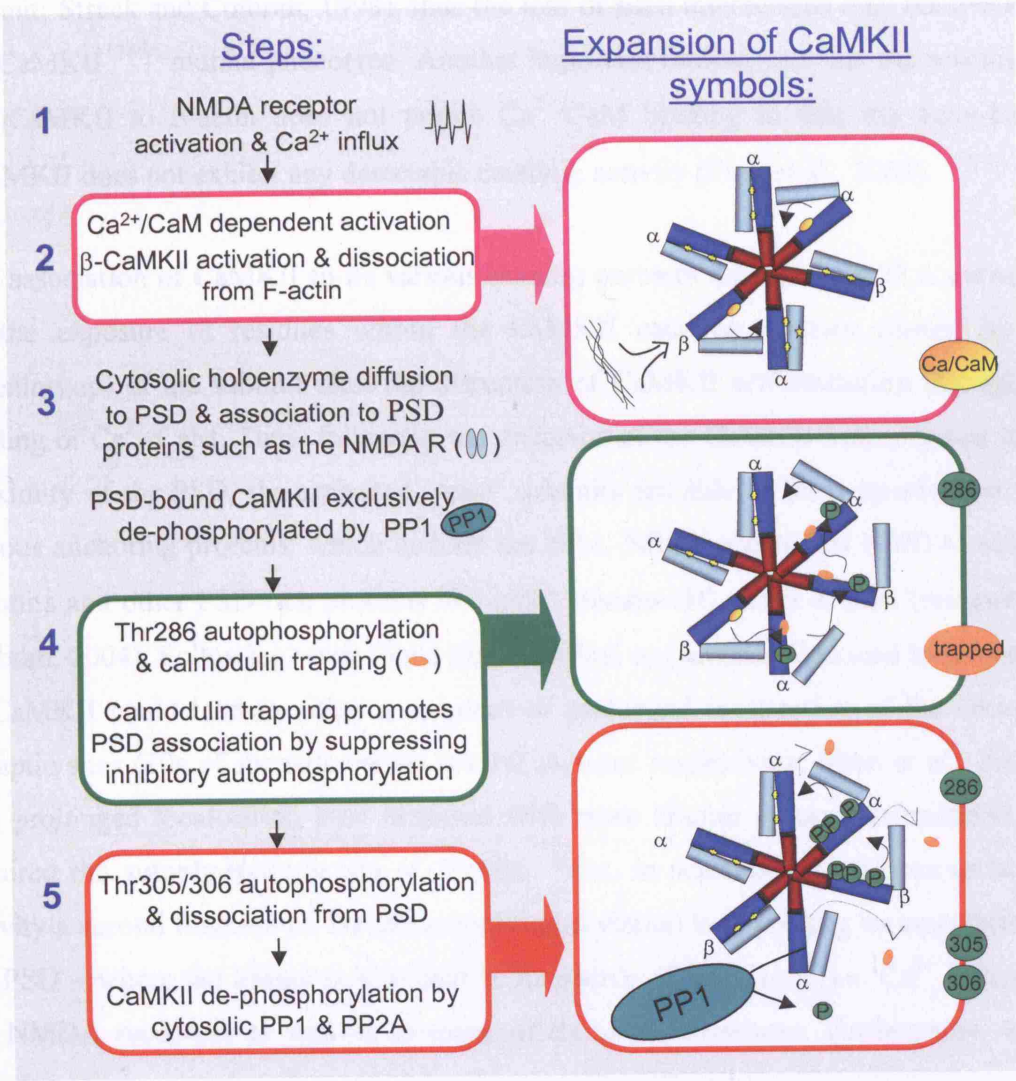
Figure 1.6 CaMKII Phosphorylation States and Regulated Translocation.

This schematic summarises the temporal sequence of the events (steps 1 – 5) that bring about the activity-dependent translocation and binding of CaMKII to the PSD as well as those that control its subsequent release. Within the dendritic spine head, the distinct CaMKII activity states are depicted as different coloured boxes with various associated symbols indicating the presence of bound or trapped Ca^{2+}/CaM and the autophosphorylation state of the kinase. The three activity states (steps 2, 4 & 5) are shown in more detail underneath.

Inactive α/β-heteromeric CaMKII holoenzymes bind to F-actin within the neuronal cytoskeleton. Following a rise in intracellular Ca^{2+} concentration (due to, for example, the influx of Ca^{2+} into the cell through NMDA receptors; **1**), CaMKII holoenzymes can be released from their cytoskeletal tether if the F-actin bound β-subunits are activated by Ca^{2+}/CaM (**2**). The now cytosolic enzyme is then able to translocate to the PSD where it may bind to various proteins, including the NMDA receptor. Within the PSD CaMKII is in an ideal location to respond to further Ca^{2+} influxes through the NMDA receptor and is in close proximity to its synaptic protein substrates (**3**). The translocation and association of CaMKII to the PSD is therefore Ca^{2+}/CaM-dependent. Within the PSD, CaMKII is thought to be dephosphorylated solely by PP1 and the high concentrations of CaMKII that can be attained within the PSD are thought to saturate PP1 activity and play an important role in the prolonged maintenance of Thr286 autophosphorylation.

Inhibitory autophosphorylation may play a role in the promotion of CaMKII dissociation away from the PSD (**5**), although the underlying mechanism for such a process is not yet known. Thr286 autophosphorylation and CaM-trapping (**4**) may therefore play an important role in the regulation of CaMKII sub-cellular location by suppressing inhibitory autophosphorylation at residues Thr305/306. Once dissociated, cytosolic CaMKII is also prone to dephosphorylation by other protein phosphatases and inactive β-subunits can re-associate with F-actin.


Ca2+ 1
NMDA receptor
Ca/CaM
Auto-P
4
T286
PP1
The PSD
De-P
3
5
Ca/CaM
De-P
T286
T305/6
Ca2+/CaM
2
De-P
Inactive CaMKII
holoenzyme
Dendritic spine
F-actin


Steps:
Expansion of CaMKII symbols:
1
NMDA receptor activation & Ca2+ influx
2
Ca2+/CaM dependent activation β-CaMKII activation & dissociation from F-actin
3
Cytosolic holoenzyme diffusion to PSD & association to PSD proteins such as the NMDA R
PSD-bound CaMKII exclusively de-phosphorylated by PP1
4
Thr286 autophosphorylation & calmodulin trapping
Calmodulin trapping promotes PSD association by suppressing inhibitory autophosphorylation
5
Thr305/306 autophosphorylation & dissociation from PSD
CaMKII de-phosphorylation by cytosolic PP1 & PP2A
Ca/CaM
286
trapped
PP1
305
306

The same group went on to show that either electrical or NMDA receptor stimulation would cause both cytosolic and F-actin bound CaMKII to translocate and accumulate at synaptic sites and that this was a Ca^{2+}/CaM-mediated process (Shen and Meyer, 1999). With respects to the βCaMKII subunit, the binding of Ca^{2+}/CaM to the subunit's autoinhibitory domain competes with and displaces its association with F-actin, thus freeing the holoenzyme from its cytoskeletal tether. This then permits the kinase to translocate to the PSD by diffusion. Ca^{2+}/CaM binding was found to be both necessary and sufficient for CaMKII (diffusion-driven) translocation and it is also independent of Thr286 autophosphorylation (Shen and Meyer, 1999). This was an important finding regarding the $CaMKII^{T286A}$ mutant mouse, as it implied that its phenotypes are not simply caused by the blockade of activity-dependent CaMKII translocation. Note, however, that the association of CaMKII to some PSD proteins *in vitro* does require that the kinase is autophosphorylated at Thr386 (for example, the NMDA receptor NR2B subunit; Strack and Colbran, 1998); thus the loss of such interactions may contribute to the $CaMKII^{T286A}$ mutant phenotype. Another important finding was that the association of βCaMKII to F-actin does not mimic Ca^{2+}/CaM binding in that the actin-bound βCaMKII does not exhibit any detectable catalytic activity (Fink et al., 2003).

The association of CaMKII to its various binding partners within the PSD is permitted by the exposure of residues within the CaMKII catalytic domain caused by the 'opening-up' of the subunit after the disruption of CaMKII self-inhibition through the binding of Ca^{2+}/CaM. Thus, following the diffusion of the CaMKII holoenzymes to the proximity of the PSD, the activated 'open' subunits are able to form interactions with various anchoring proteins, which include the NR1, NR2A and NR2B NMDA receptor subunits and other PSD rich proteins like p190, densin-180 and α-actinin (reviewed in Colbran, 2004). Following work found that electrical or glutamate-induced translocation of CaMKII could lead to either a transient or prolonged localization of the kinase at synaptic sites (10s of seconds versus several minutes respectively; Shen et al., 2000a). The prolonged localization only occurred with more intense synaptic stimulation and required the autophosphorylation of Thr286. Thus, in addition to autonomous kinase activity a second function of Thr286 autophosphorylation is to prolong its association to the PSD – where the kinase is also near to its source of activation (i.e. Ca^{2+} influx via the NMDA receptor) as well as to many of its target substrates. Furthermore, when

Thr286 autophosphorylation was mimicked by the replacement of Thr286 with an aspartate residue, the kinase took 10-times longer to dissociate from synaptic sites, indicating that dephosphorylation of the autophosphorylated Thr286 is required to initiate kinase dissociation (Shen et al., 2000a). Indeed pharmacological inhibition of PP1 caused a similar prolongation of CaMKII association to synaptic sites, as did the application of non-hydrolysable cAMP, which presumably promoted the Thr286 autophosphorylated state of CaMKII through the PKA-dependent phosphorylation of inhibitor-1 and thus reduced PP1 activity (Shen et al., 2000a).

Moreover, the induction of NMDA receptor-dependent chemical LTP in organotypic hippocampal slice cultures was found to produce a translocation of GFP-tagged CaMKII to synaptic sites within dendritic spines that could persist for at least 90 minutes (Otmakhov et al., 2004). This indicates that there must be specific conditions and factors that contribute to the control of the persistence of CaMKII redistribution in order to result in the three levels that have been observed: the transient and relatively prolonged forms that respectively persist over timescales of seconds and several minutes (Shen et al., 2000a), and a more persistent form that can be maintained over a timescale of hours (Otmakhov et al., 2004).

Interestingly, it seems that the dissociation of CaMKII is also promoted by autophosphorylation, but of the inhibitory autophosphorylation sites at Thr305 and Thr306 (which suppress the rebinding of released CaM), as the mutation of these sites to non-phosphorylatable alanine residues resulted in much slower dissociation rates of the GFP-tagged kinase (Shen et al., 2000a). This finding agreed with a previous *in vitro* study that found CaMKII autophosphorylated at Thr305 and Thr306 has reduced affinity for the PSD (Strack et al., 1997b). Consistent with this, in transgenic mice where both Thr305 and Thr306 of the αCaMKII subunit were mutated to non-phosphorylatable amino acids (alanine (A) and valine (V) respectively; TT305/306VA), isolated PSDs contained much higher levels of CaMKII; whereas mimicking autophosphorylation at just one of the residues by mutating it to an aspartate (T305D; which prevents the activation of the subunit by Ca^{2+}/CaM) dramatically reduced the association of CaMKII with the PSD (Elgersma et al., 2002). The mechanism for this Thr305/306 autophosphorylation-promoted dissociation of CaMKII is not yet clear. It

may be that the association of CaMKII to some of its binding partners within the PSD requires that CaMKII is in a CaM-bound state, thus inhibiting the rebinding of Ca^{2+}/CaM following inhibitory autophosphorylation may promote its dissociation away from the synapse (Colbran, 2004).

Elgersma et al. (2002) also investigated the effects of the Thr305/306 mutations upon synaptic plasticity, learning and memory in the transgenic mice. The stimulation threshold for LTP induction at CA1 synapses was reduced in the mice with blocked inhibitory autophosphorylation (TT305/306VA) such that synaptic potentiation could be readily induced with stimulation intensities that were insufficient in wild-type mice. While these transgenics were able to learn the location of a hidden platform in the Morris water maze, they were unable to re-learn a second location indicating impaired learning mechanisms. Similarly, the mice performed equally as well as control wild-types in tests of contextual fear conditioning, but the transgenics were unable to differentiate between the conditioned context and a novel context, exhibiting fear responses to both. These experiments indicated that the facilitation of LTP induction (resulting from blocked inhibitory autophosphorylation and enhanced association of CaMKII to the PSD) culminates in more rigid and less finely tuned learning in the TT305/306VA mice.

In contrast, the mice possessing the T305D mutation and decreased levels of PSD-associated CaMKII, showed no LTP at CA1 synapses and were unable to form long-term memories in either the Morris water maze or contextual fear conditioning paradigm (Elgersma et al., 2002). These results support the view that the activation and translocation of CaMKII holoenzymes to the PSD is critical for synaptic plasticity and for the endogenous processes that underlie learning and memory in the brain.

With respects to the $CaMKII^{T286A}$ mutant mice, in addition to the loss of autonomous kinase activity, the loss of Thr286 autophosphorylation-dependent CaM-trapping means that the kinase's built-in mechanism of delaying the onset of inhibitory autophosphorylation at Thr305 and Thr306 and CaMKII dissociation away from activated synapses is also lost. This impairment is therefore likely to have contributed to the synaptic and behavioural phenotypes seen in the $CaMKII^{T286A}$ transgenics.

1.10.7 CaMKII Binding to the NMDA Receptor

A wealth of biochemical data exists to support the notion that when targeted to the PSD a primary binding partner of CaMKII is the NMDA receptor (Leonard et al., 1999;Bayer et al., 2001). Various *in vitro* binding studies have found that the NR2A, NR2B and NR1 NMDA receptor subunits are all capable of binding to CaMKII (e.g. Gardoni et al., 1998;Strack and Colbran, 1998;Gardoni et al., 1999;Leonard et al., 1999;Strack et al., 2000;Gardoni et al., 2001b;Leonard et al., 2002). Some studies favour the cytoplasmic tail of the NR2B subunit as the primary target for CaMKII interaction (Strack and Colbran, 1998;Strack et al., 2000), but strong associations with the C-terminus of the NR1 subunit are also convincing (Leonard et al., 1999;Leonard et al., 2002). It is possible that if CaMKII is primarily targeted and binds to a particular subunit, when bound the CaMKII holoenzyme may then also be able to make secondary interactions with the other subunits present in the heterotetrameric NMDA receptor. It is also important to bear in mind that although in some studies CaMKII may be shown to associate to various NMDA receptor-derived peptides, this does not necessarily mean that these regions of the receptor are also able to bind to CaMKII *in situ* when all other interacting proteins within the PSD are also present. It is agreed however that CaMKII must be activated by Ca^{2+}/CaM in order to associate with the NMDA receptor. Some studies indicate that Thr286 autophosphorylation is also required (Gardoni et al., 1998;Strack and Colbran, 1998;Gardoni et al., 1999;Leonard et al., 1999;Strack et al., 2000), but another study did not find this to be necessary (Bayer et al., 2001). Nonetheless, it is clear that the interaction between CaMKII and NMDA receptors is at least strongly enhanced by Thr286 autophosphorylation, most probably due to CaM-trapping as discussed above.

The study by Bayer et al. (2001) is particularly interesting because their findings suggest that the binding of CaMKII to the NMDA receptor may affect CaMKII activity states. They proposed that there are at least two sites within the cytoplasmic tail of the NR2B subunit that may bind to the kinase: 1) a site encompassing residues 1120-1482 that can bind to the CaMKII Ca^{2+}/CaM-activated kinase without the requirement for prior Thr286 autophosphorylation (deduced as they observed binding in the absence of ATP and with the T286A mutant CaMKII); and 2) a site encompassing residues 839-1120 that only binds Thr286 autophosphorylated CaMKII. The interacting domain lying

within the first region was identified as sharing considerable sequence similarity to a region within the autoinhibitory domain of CaMKII (i.e. the domain that binds to and inhibits the catalytic domain under basal conditions) and in combination with the fact that it did not require Thr286 autophosphorylation was thus considered to be of particular interest. Moreover, specific peptides that include this region of the NR2B subunit had previously been found to be effective at competing with NR2B for CaMKII binding, thus supporting the hypothesis that CaMKII may bind to the NMDA receptor within this region *in vivo* (Leonard et al., 1999;Strack et al., 2000). Bayer et al. (2001) proposed that this sequence of this segment of the NR2B subunit C-terminal would be able to mimic the portion of the autoinhibitory domain that surrounds the Thr286 but that it would not mimic the pseudosubstrate region. Thus the interaction of CaMKII to this region of the NR2B subunit might be able to functionally mimic Thr286 autophosphorylation by blocking CaMKII autoinhibition by associating to the T-site, whilst leaving substrate binding and catalytic activity at the S-site unimpaired.

Indeed, Bayer et al. went on to demonstrate that when CaMKII bound to a peptide made-up of the NR2B residues 1120-1482, the interaction between CaMKII and the peptide permitted kinase activity to continue once Ca^{2+}/CaM had dissociated from the activated kinase. This activity state was transient, lasting between seconds and minutes. However, if this interaction also occurs between CaMKII and the intact NR2B-subunit *in vivo* it signifies a novel form of autonomous Ca^{2+}/CaM-independent CaMKII activity that does not require any previous Thr286 autophosphorylation. Although short-lasting it could have important physiological consequences because this form of autonomous activity would not be reversed by phosphatase activity. It would however, only be relevant for the specifically NR2B-bound subunits within each holoenzyme, but this might still result in an enhancement of holoenzyme function as it would remove the requirement for the coincident binding of two Ca^{2+}/CaM molecules to adjacent subunits to induce Thr286 autophosphorylation; the binding of a single Ca^{2+}/CaM molecule to the subunit adjacent to the NR2B-bound active subunit would be sufficient (Bayer et al.., 2001).

Bayer et al. also found that the binding of CaMKII to this NR2B peptide led to the trapping of Ca^{2+}/CaM, again mimicking the consequences of Thr286-

autophosphorylation. Once more, if this also occurs *in vivo* then it could also lead to CaM-trapping induced suppression of inhibitory autophosphorylation at Thr305/306 and the subsequent prolonged association of CaMKII to the PSD.

Note, various other proteins within the PSD are also able to interact with the CaMKII holoenzyme, thus the binding to the NMDA receptor may not be the only mode of association to the postsynaptic membrane (reviewed in detail in Colbran, 2004).

1.10.8 CaMKII as a Frequency Detector of Ca^{2+} Oscillations

One way in which the pattern of presynaptic firing (that makes up the 'neural code') can convey information in the postsynaptic cell is through the dynamics of the Ca^{2+} signals that are produced. The characteristics of such signals like the frequency, amplitude and kinetics of Ca^{2+} oscillations/spikes, will be primarily shaped by the pattern of synaptic activity, by the properties of the various postsynaptic Ca^{2+} conductances (including the release of Ca^{2+} from intracellular stores) and factors that alter the decay times of Ca^{2+} transients. As Ca^{2+} tranisents are able to trigger a vast array of distinct processes, cells must express local postsynaptic molecular decoders that are capable of translating such Ca^{2+} signals into the downstream signalling events that lead to specific neural consequences. It can be further hypothesised that such Ca^{2+} sensors might have the capacity to be 'tuned' in order to respond preferentially to certain patterns of Ca^{2+} influx over others.

Computer simulations of CaMKII activity, that consider its physical, catalytic and regulatory properties, have predicted that this kinase would be able to translate the frequency of repetitive Ca^{2+} spikes in neurones (and therefore transients of CaM activation) into distinct levels of kinase activity (Hanson et al., 1994;Dosemeci and Albers, 1996). Such models firstly predict that a *threshold frequency* must exist that can decode a Ca^{2+} signal into a minimal level of autonomous CaMKII activity. This critical frequency must be one that results in a rise of Ca^{2+} that can enable at least one subunit to become autophosphorylated at Thr286 through the coincident binding of Ca^{2+}/CaM molecules to two adjacent CaMKII subunits. The dissociation rate of Ca^{2+}/CaM from CaMKII would be an important element in determining this frequency threshold.

Secondly, as frequencies increase above this threshold, increased levels of autonomy should be produced. This would be because: a) the probability of coincident binding and thus of Thr286 autophosphorylation would increase, hence reducing the significance of this initial limiting factor; b) now one additional Ca^{2+}/CaM is required rather than two to produce further autophosphorylation (which also means that a lower level of Ca^{2+} compared to that required for initiation may be sufficient to enable the propagation of Thr286 autophosphorylation around the holoenzyme ring to proceed) and c) autophosphorylation of one subunit would facilitate the autophosphorylation of a second subunit during subsequent spikes in a feed-forward cooperative manner because one autonomous subunit can phosphorylate its neighbouring Thr286 without the need for CaM to be still bound (Hanson et al., 1994). This functional cooperative nature of CaMKII holoenzyme autophosphorylation would enhance the integration of subsequent Ca^{2+} pulses in a train and enable the enzyme to produce relatively greater levels of kinase activity across increasing frequencies.

The sensitivity of CaMKII to the frequency of Ca^{2+} oscillations was investigated *in vitro* by De Koninck and Schulman (1998). In agreement with the above predictions, they found that both α and β CaMKII homomeric holoenzymes could decode the frequency of delivered Ca^{2+} pulses into distinct amounts of autonomous kinase activity (used as a measure of Thr286 autophosphorylation), with higher frequencies producing higher levels of autonomy. The potential cooperative nature of CaMKII autophosphorylation was also investigated by De Koninck and Schulman (1998). As expected, it was found that if CaMKII already exhibited a low level of autonomy before the application of the Ca^{2+} pulses of a particular frequency, greater levels of autonomy were produced compared to that of initially inactive enzyme. Such pre-autophosphorylated CaMKII would also show increased sensitivity to lower frequencies of stimulation (De Koninck and Schulman, 1998). Thus an important feature of CaMKII function may be in its ability to produce distinct levels of Thr286 autophosphorylation in response to particular Ca^{2+} signals depending upon its recent history of activation; i.e. to be able to integrate present with past levels of synaptic activity into specific states of Thr286 autophosphorylation.

More recent work has added to the value of these results by showing that the range of

Ca^{2+} concentrations over which CaMKII can produce discrete levels of autonomous activity is physiologically relevant (Bradshaw et al., 2003). For example, under their *in vitro* conditions, CaMKII was only 10% autophosphorylated at 1 μM Ca^{2+} but 88% autophosphorylated at 1.8 μM Ca^{2+}. This study concluded that the ultra-sensitivity of CaMKII to Ca^{2+} arose from two cooperative and Ca^{2+}-dependent interactions: the cooperative binding of Ca^{2+} molecules to Ca^{2+}/CaM (Chin and Means, 2000) and, as also discussed above, the requirement for coincident binding of Ca^{2+}/CaM to two adjacent CaMKII subunits in order to produce Thr286 autophosphorylation.

As predicted by De Koninck and Schulman the frequency sensitivity of CaMKII was also affected by the duration of Ca^{2+} pulses; presumably because longer durations enhanced the probability of coincident binding of Ca^{2+}/CaM on neighbouring subunits within a given pulse, affecting both the critical frequency threshold and integration properties of CaMKII.

As there are many neuronal factors that can affect the temporal as well as spatial dynamics of local Ca^{2+} signals (Augustine et al., 2003), the sensitivity of CaMKII to Ca^{2+} oscillations may provide one way through which the triggered modulation of Ca^{2+} dynamics is transduced into alterations of the downstream signalling cascades. For example, the activity-dependent modulation of CaM availability by PKC-mediated phosphorylation of neuromodulin or neurogranin (Apel et al., 1990;Ramakers et al., 1999) could directly affect the frequency response of CaMKII. Moreover, the ability of a neurone to modulate the properties of CaMKII itself would provide another powerful mechanism by which it could adjust its responses to specific Ca^{2+} signals. One mode could be through the regulation of the holoenzyme's isoform composition as biochemical studies have shown that the affinity of βCaMKII for Ca^{2+}/CaM is much greater than that of αCaMKII (Hanson and Schulman, 1992;De Koninck and Schulman, 1998;Brocke et al., 1999). The isoforms also show different rates of translocation to the PSD (quickest for -homomeric CaMKII, slowest for - homomeric CaMKII, and at an intermediate rate for an / -heteromer; Shen and Meyer, 1999) which may in turn be able to affect the activation of the kinase by subsequent Ca^{2+} spikes due to its relative proximity to the source of Ca^{2+} influx via the NMDA receptor. Differential rates of translocation could also affect the temporal aspects of activated kinase function due to

the speed of its access to substrates within the PSD or even affect the dephosphorylation rate of the kinase that is thought to be restricted within the specialized environment of the PSD (Strack et al., 1997a;Lisman and Zhabotinsky, 2001).

In accordance with the differential sensitivities of α and β isoforms to Ca^{2+}/CaM, De Koninck and Schulman (1998) showed that the frequency response curves for the two homomeric holoenzymes were very different, with βCaMKII possessing a lower critical frequency for activation and exhibiting much greater levels of autonomy at all frequencies tested compared to αCaMKII. As expected, the frequency response curve for α/β heteromeric CaMKII was intermediate to that of the two homomers, supporting their hypothesis that the differential expression of the two isoforms at a given site would be able to modulate the frequency response of CaMKII to Ca^{2+} oscillations (De Koninck and Schulman, 1998).

The distinct sensitivities of homomeric and heteromeric CaMKII to Ca^{2+}/CaM were also demonstrated by Brocke and colleagues (1999); their experiments further revealed that the subunit composition of the holoenzyme is determined by the relative expression level of each isoform at a given site within a cell (Brocke et al., 1999). Thus, the regulated synthesis of the two isoforms provides one way through which cells could tune the composition of their CaMKII holoenzymes in order to be able to respond optimally to specific frequencies or amplitudes of Ca^{2+} signals. Indeed recent work in cultured hippocampal neurones found that changes in the relative expression levels of the α and β isoforms could be dictated by the history of synaptic activity, indicating that neurons may possess such a tuning ability (Thiagarajan et al., 2002). Furthermore, it has been shown that the different splice variants of the β-isoform can generate different levels of autonomy in response to Ca^{2+} oscillations *in vitro* (despite exhibiting comparable levels of activity towards an exogenous peptide substrate and similar maximal levels of autonomy; Bayer et al., 2002). The activity-driven control of alternative splicing of the individual isoforms may also therefore provide a means by which neurones can tune their responses to specific Ca^{2+} signals.

In summary, the overall properties of a CaMKII holoenzyme reflects the combination of the individual properties of each composite subunit and mechanisms that control the

kinase composition may therefore provide a powerful means through which a neurone can tune the CaMKII to respond to specific Ca^{2+} signals. Modulating the sensitivity of CaMKII holoenzymes to Ca^{2+} signals may enable neurones to reset the patterns/levels of synaptic activation that are required to trigger LTP expression. This might present a mechanism underling metaplasticity, or even underlie certain homeoplastic neuronal responses.

1.10.9 The Persistence of CaMKII Thr286 Autophosphorylation and Activity during LTP Maintenance

Although it is widely accepted that increased CaMKII activity and the autophosphorylation of Thr286 are both critical for the initial expression of LTP (for instance, through the modification of AMPA receptor responses) as well as for learning and memory, a question that is still of great debate and remains unresolved is whether CaMKII activity is also required for the maintenance of LTP (Chen et al., 2001;Lengyel et al., 2004). Several lines of evidence have fuelled the hypothesis that LTP might require the persistent activation of CaMKII. 1) First of all, it has been shown by several groups that the induction of LTP is associated with a significant and persistent increase in the levels of Thr286 autophosphorylation as well as the persistent phosphorylation of endogenous substrates of CaMKII (typically assessed at 30 or 60 minutes post LTP induction; e.g. Fukunaga et al., 1993b;Fukunaga et al., 1995;Ouyang et al., 1997;Barria et al., 1997b;Makhinson et al., 1999;Lee et al., 2000;Lengyel et al., 2004); both of which suggest maintained CaMKII activity during at least the early phases of LTP expression. Note, these observations also encouraged the further development of a now long-standing theoretical model of LTP where the switching of CaMKII into a persistently Thr286 autophosphorylated 'on' state is proposed to underlie both the all-or-nothing nature of LTP and its persistence (Lisman, 1985;Lisman and Goldring, 1988;Lisman, 1989;Zhabotinsky, 2000;Okamoto and Ichikawa, 2000b;Lisman and Zhabotinsky, 2001). This 'on' state of CaMKII is defined by its theoretical capacity to remain in a stable and highly Thr286 autophosphorylated state even when increased Ca^{2+} levels within the neurone have returned to baseline. The model puts forward that the high concentrations of translocated CaMKII that are observed within the PSD and the ability of CaMKII to self-phosphorylate to produce autonomous activity will enable the kinase to resist inactivation by PP1 due to phosphatase saturation; PP1 activity will

be limited by the number of its scaffold binding sites within PSD and by its activity-dependent inhibition as discussed above. More recently it has also been pointed out that the ability of CaMKII to autophosphorylate at other sites within the kinase, where phosphorylation does not require prior Ca^{2+}/CaM binding or autophosphorylation at Thr286 (Lengyel et al., 2000;Bayer et al., 2001), may also aid the production of a hyper-phosphorylated kinase that can saturate PP1 (Lengyel et al., 2004). Furthermore, the CaMKII switch model poses that high levels of autophosphorylation would also be maintained even when faced with protein turnover and the insertion of new inactive and un-phosphorylated holoenzymes as long as PP1 activity remained saturated. In this case, the presence of highly phosphorylated holoenzymes would cooperatively aid the insertion and phosphorylation of the new subunits by being the shared target of saturated PP1 activity (Lisman and Zhabotinsky, 2001).

2) A second line of evidence in support of the proposition that CaMKII may be required for LTP maintenance has come from the recent observation that NMDA receptor-dependent LTP can trigger the persistent increase in the concentration of CaMKII within dendritic spines (Otmakhov et al., 2004); a finding that is also likely to be linked with persistent Thr286 autophosphorylation which, as discussed earlier, prolongs the association of translocated CaMKII to synaptic sites (Shen et al., 2000a). 3) Finally, LTP has also been associated with the activity-dependent increase in the total levels of CaMKII protein within the activated dendrites of CA1 neurones in hippocampal slices (Ouyang et al., 1997) – an increase that is driven by the local translation of dendritic αCaMKII mRNA into protein occurring as soon as 5 minutes after LTP induction (Ouyang et al., 1999). Indeed, LTP has also been found to trigger the recruitment of CaMKII mRNA and polyribosomes (necessary for mRNA translation) into dendritic spines (Ostroff et al., 2002;Havik et al., 2003). Moreover, preventing the dendritic translation of αCaMKII in transgenic mice by disrupting the signal for mRNA localisation to the dendrites caused a significant decrease in late-phase LTP in CA1 neurones and resulted in impairments in several forms of hippocampal-dependent memory (Miller et al., 2002). Although the work by Ouyang et al. (1999) suggested that the dendritic synthesis of CaMKII may occur within just a few minutes of LTP induction, the findings from the transgenic mice of Miller et al. (2002) indicate that the local synthesis of αCaMKII on a short time-scale is not necessary for the early phase of

LTP expression, since the early phase of synaptic potentiation was indistinguishable between wild-types and mutants. The transgenics also showed a dramatic reduction in total αCaMKII protein levels under baseline conditions; the levels of CaMKII in whole hippocampal homogenates from the mutants were only 44% of the wild-type levels, and in the PSD fractions CaMKII levels stood at only 17% compared to wild-types; levels of βCaMKII were unaltered (Miller et al., 2002). Although this reduction did not appear to impair the induction of LTP it could potentially have contributed to the impaired long-term maintenance of LTP at Schaffer collateral inputs. Nevertheless, this study does make a strong case for the involvement of synaptic CaMKII during the maintenance phases of LTP at excitatory synapses in the hippocampus and most importantly for the endogenous processes that underlie hippocampal-dependent memories in the brain. Furthermore, heterozygote mice for the αCaMKII null mutation which have half the normal levels of αCaMKII have been found to exhibit normal learning and memory in two of the behavioural tests used by Miller and colleagues (Frankland et al., 2001) . They also show normal late LTP which supports the hypothesis that the specific loss of dendritic translation in the above transgenics is likely to be the detrimental factor rather than reduced baseline levels.

More recently, evidence has also suggested that the activity-driven synthesis of dendritic αCaMKII may even be dependent upon CaMKII catalytic activity itself. Atkins and colleagues found that the mRNA translation factor – cytoplasmic polyadenylation element binding protein (CPEB), that initiates mRNA translation – is phosphorylated (and thus activated) by CaMKII but not by other kinases known to be activated during LTP (Atkins et al., 2004). Moreover, only stimulation protocols that evoke the late-stage persistent forms of LTP (> 3 hours) resulted in CPEB phosphorylation, whereas synaptic potentiation that only persists for 1-3 hours did not (Atkins et al., 2005). These findings fit with the observations made by Miller et al. (2002) that the local *de novo* synthesis of CaMKII is only required for the more long-lasting forms of synaptic potentiation.

Despite the above lines of evidence that suggest that CaMKII may be involved or even required for the maintenance of LTP, the majority of studies that have attempted to impair LTP expression by the application of CaMKII inhibitors on pre-established LTP

in vitro have failed to observe an effect (Malenka et al., 1989;Malgaroli et al., 1992;Hvalby et al., 1994;Otmakhov et al., 1997;Chen et al., 2001;Yang et al., 2004). One study did successfully show that LTP in the hippocampus could be reversed by the application of a CaMKII inhibitor (Feng, 1995), however an attempt to repeat this work using similar experimental conditions failed to see any effect (Chen et al., 2001). In fact the study by Chen et al. (2001) went to some lengths to address whether various methodological factors may be hindering the reversal of LTP by the inhibition of CaMKII activity. They assessed whether CaMKII inhibitors may be unable to access the kinase and block its catalytic activity whilst it is associated to the PSD and whether the lack of reversal could be due to impaired phosphatase activity under experimental conditions. They found that the ability of both soluble and PSD-bound CaMKII to phosphorylate an exogenous synthetic substrate of CaMKII *in vitro* was potently inhibited by the CaMKII inhibitor AC3-1. However, further analysis in whole brain extracts revealed that the potency of inhibitor was significantly reduced for some protein targets of CaMKII. In particular the potency of CaMKII inhibition towards the phosphorylation of a protein complex that corresponded to the NR2B subunit of the NMDA receptors and densin-180 was significantly less than the potency of the inhibitor towards soluble fractions of the brain extract which included the autophosphorylated forms of α- and βCaMKII. Although, high concentrations of the inhibitor were used in the LTP experiments, it may still be possible that LTP could persist because a critical level of CaMKII activity within the PSD persisted due to inefficient access of the inhibitor.

They also reported (but did not show) that LTD could still be induced under their hippocampal slice and recording conditions, showing that the lack of LTP reversal could not be explained by the wash-out of protein phosphatases in the whole-cell recordings. Furthermore, when they increased Ca^{2+} levels in the whole-cell pipette to just above normal resting concentrations in order to aid the Ca^{2+}-dependent activation of PP2B and PP1, this was again unable to promote the reversal of LTP via the application of CaMKII inhibitors. Together, the numerous reports that LTP maintenance is not blocked by the inhibition of CaMKII unexpectedly suggest (considering the other lines of evidence) that the catalytic activity of this kinase may not be required for the

maintenance of LTP expression.

Chen et al. (2001) also suggest a possible explanation for the CaMKII inhibitor-mediated reversal of LTP that was observed by Feng (1995). They suggest that the successful reversal may be the consequence of artificially elevated levels of intracellular Ca^{2+} that could have resulted by Feng's use of relatively low-resistance microelectrodes (50 MΩ) to impale CA1 cells. The use of this method to record from and deliver drugs to the inside of CA1 neurones may have led to the influx Ca^{2+} into the cell due to the sharp electrodes not forming a tight enough seal with the membrane. Thus Ca^{2+}-stimulated mechanisms that promote synaptic de-potentiation/depression may already have been active and thus more readily able to reversal LTP once the balance between kinase and phosphatase activities was tipped just a bit more in favour of the phosphatases via CaMKII inhibition. Until the reversal of LTP by CaMKII inhibitors can be repeated this single study by Feng (1995) does not hold weight.

The inability to impair the expression of pre-established LTP via the blockade of CaMKII activity seems to stand in contrast with observations of increased and persistent Thr286 autophosphorylation and up-regulated protein synthesis that have been found to accompany LTP. However, some of the findings from the theoretical studies that have modelled the ability of CaMKII to remain in a long-lasting highly autophosphorylated and stable 'on' state within the PSD once Ca^{2+} has returned to baseline levels (Zhabotinsky, 2000;Lisman and Zhabotinsky, 2001) may provide a possible explanation for this discrepancy. In particular, these models suggest that the rate of dephosphorylation and subsequent re-phosphorylation of CaMKII subunits would be very slow. This is because both processes would be Ca^{2+}-dependent and as basal levels of Ca^{2+} in CA1 neurones are very low, the rate of such Ca^{2+}-dependent processes would be very slow. The inter-subunit autophosphorylation of Thr286 must occur in a Ca^{2+}-dependent manner because it is known that the substrate CaMKII subunit requires that Ca^{2+}/CaM is bound in order to expose the Thr286 residue for phosphorylation by its active neighbour (Hanson et al., 1994). The Lisman and Zhabotinsky model of a persistent Thr286 autophosphorylated CaMKII state assumed, however, that the activity exhibited by the subunit that catalyses the phosphorylation of its neighbour's Thr286 would be Ca^{2+}-independent, i.e. autonomous. However, it was recently reported that the

increased levels of CaMKII autonomous activity that follow LTP induction *in vitro* return to basal levels within 5 minutes (Lengyel et al., 2004; but see Fukunaga et al., 1993b). Despite this, if autonomous activity is required for the re-phosphorylation of Thr286 residues on neighbouring subunits it must be assumed that these basal levels would be sufficient since persistent Thr286 autophosphorylated CaMKII is observed after LTP induction. The basal levels of autonomous CaMKII activity were calculated by Lengyel et al. (2004) to stand at approximately 11% of the total kinase activity that can be induced in the presence of Ca^{2+}/CaM.

With respects to PP1, its activity is also Ca^{2+}-dependent because it requires Ca^{2+}/CaM dependent PP2B activity to relieve it from inhibitor-1 inhibition. Thus low Ca^{2+} levels would also dictate that PP1 availability is limited. In support of the model's prediction that slow rates of Thr286 de- and re-phosphorylation would underlie the maintenance of persistently autophosphorylated CaMKII during LTP, measurements from hippocampal slices have indeed shown that CaMKII is dephosphorylated very slowly under basal conditions (Gardoni et al., 2001a). Lisman and Zhabotinsky suggest that the persistent Thr286 autophosphorylated 'on' state of this proposed CaMKII bi-stable switch would exist in a nearly frozen state, which would enable the persistence of Thr286 autophosphorylation to be maintained in an energy-efficient and, therefore, viable manner.

If the re-phosphorylation of Thr286 is an important process for LTP maintenance and as suggested above only requires very low levels of both Ca^{2+}/CaM-dependent and autonomous CaMKII activity to maintain sufficient levels of Thr286 autophosphorylation to resist PP1 activity, then it may be possible that such low levels of kinase activity can also persist despite the presence of CaMKII inhibitors which may also exhibit reduced potencies within the PSDs of postsynaptic spines (Chen et al., 2001). It would be very interesting to know, therefore, if and to what extent the application of CaMKII inhibitors to potentiated synapses affects the relative level of CaMKII accumulation and Thr286 autophosphorylation within the PSD. If little change is observed then it can be assumed either that the re-phosphorylation of Thr286 is not substantially affected by the inhibitor or that rates of de-phosphorylation are so slow that the run-down of LTP by CaMKII inhibition is not detected within the time-scale of

the experiments.

Note, the short-lived nature of increased levels of autonomous CaMKII activity following LTP induction (i.e. no longer than 5 minutes; Lengyel et al., 2004) was a surprising discovery because it implies that despite the persistly high levels of Thr286 autophosphorylated kinase, the ability of these subunits to exhibit autonomous activity is somehow lost (Lengyel et al., 2004). An explanation for this observation remains unsolved. As suggested by Lengyel and colleagues (2004), one possibility could be that specific protein interactions formed between CaMKII and molecules within the PSD might specifically inhibit catalytic activity of the Thr286 autophosphorylated kinase. Another explanation might involve the autophosphorylation of CaMKII at residues Thr253 and/or Ser279 which have been observed to occur *in vitro* after the prolonged exposure of CaMKII to Ca^{2+}/CaM and which have been shown to be accompanied by a parallel decrease in enzyme activity (Dosemeci et al., 1994;Lengyel et al., 2000). The short-lived nature of increased autonomy might also, therefore, reflect a side effect of the LTP chemical induction protocol used by Lengyel et al. that involved a 10 minute incubation in the K^+ channel blocker TEA to depolarise large numbers of neurones within an acute slice.

1.11 Environmental Enrichment

As outlined in the first section of this introduction, the overall aim of experiments reported within this thesis was to uncover evidence of endogenous forms of synaptic plasticity in the mouse hippocampus by comparing the synaptic and structural properties of CA1 pyramidal neurones between wild-type and $\alpha CaMKII^{T286A}$ mice. In an attempt to increase the magnitude and thus the probability of detecting any differences between wild-type and mutant neurones, the animals were raised in environmentally enriched housing in order to increase the opportunity for experience-mediated synaptic plasticity to take place in the hippocampus.

The concept of an 'enriched environment' was first posed by Donald Hebb after he discovered that the rats he took home as pets for his children that were freely allowed to explore his house (both the rats and his children) performed substantially better in behavioural tasks than littermates that were housed solely in the laboratory. Hebb

concluded that "*the richer experience of the pet group during development made them better able to profit by new experience at maturity - one of the characteristics of the 'intelligent' human being*" (Hebb D.O., 1949). Since this time, a wealth of evidence has been presented describing the numerous effects that enrichment has upon brain biochemistry, structure and physiology as well as enhanced cognitive ability compared to animals housed in standard conditions (reviewed in: Rosenzweig and Bennett, 1996;Van Praag et al., 2000).

The main advantage of using environmental enrichment as a tool to study the plasticity of the nervous system is that because the changes occur *in vivo* and are caused by the animal's actual experience, rather than resulting from experimental manipulations (like the electrical stimulation of afferent pathways in the brain), the detectable enrichment-mediated changes offer physiologically relevant clues about the endogenous forms of plasticity that take place in the brain. In contrast, plastic changes identified to result from the induction of experimental forms of plasticity may not necessary characterise the changes that occur following experience-stimulated neural activity *in vivo*.

Some of the enrichment-mediated changes found in the brain may characterise the endogenous substrates of learning and memory in the brain, although others may simply reflect general changes that occur with increased levels of neural activity. In addition to these possibilities, the identified changes might also help understand the biological basis of the observation that enriched animals exhibit enhanced learning and memory abilities. For instance, it was found that when animals raised in enriched environments were then trained in the Morris water maze, *larger* training-induced *increases* in BDNF mRNA levels were detected in their hippocampi compared to control animals from standard housing conditions, and thus correlated with the improved spatial memory in the enriched animals (Falkenberg et al., 1992). As BDNF signalling is known to modulate synaptic and morphological plasticity in the brain (Tyler et al., 2002), these findings provide a clue towards the understanding of how the past experience of an animal can alter brain neurobiology such that future cognitive abilities are enhanced. Interestingly, BDNF has also been shown to stimulate local protein synthesis of CaMKII in neuronal dendrites (Aakalu et al., 2001), thus enhanced CaMKII signalling may even underlie enhanced enrichment-mediated learning abilities in rodents and again

supports the use of the CaMKII^{T286A} mouse in this thesis.

1.11.1 The Enriched Housing Environment

In contrast to standard laboratory rodent housing conditions, which are typically small barren cages that lack most features of a natural habitat and house 1-3 animals, enriched housing usually accommodates more animals in larger cages that contain a variety of inanimate objects, such as tunnels, ladders, toys, various nesting materials and running-wheels for voluntary exercise. Social, physical and sensory cues are all increased in the enriched housing paradigm and although some studies have attempted to dissect out which factors may play more prominent roles in promoting plastic change to occur (e.g. Schrijver et al., 2002;Lambert et al., 2005), it is generally considered that it is an interaction of all elements that leads to the changes found in the brain and in the animals' behaviours and that no one single variable can account for all consequences of enrichment. It is thought that the neural changes are likely to arise from the combination of various systemic effects driven by increased physical-activity, which may prime brain regions like the hippocampus to respond to environmental stimulation (like the release of growth factors; reviewed in Cotman and Berchtold, 2002), along with neural activity-dependent changes that are mediated by increased arousal and cognitive stimulation of the animal (reviewed in: Rosenzweig and Bennett, 1996;Van Praag et al., 2000). This latter aspect is likely to involve consequences driven from increased social interactions as well as the animals' experiences of novelty and there is a general view that many of the changes detected in brain regions like the hippocampus may specifically result from increased levels of learning and memory having taken place. Some of the evidence supporting this hypothesis is presented below.

One problem faced when considering and comparing the data from various enrichment studies is that the specifics of the enrichment protocols often differ. Differences often exist in the nature of enriched environments and how frequently they are changed in order to enhance the animals' exposure to novelty (if changed at all). Protocols also often differ in the number of days and/or hours per day that the animals are permitted to explore the enriched environments. Moreover, there are the usual problems of differences in animal species, strain and animal ages used that complicate the comparison of different studies. For example, different strains of mouse have been

found to exhibit distinct levels of LTP in response to identical induction protocols and conditions (Nguyen et al., 2000;Jones et al., 2001b); thus differences in the inherent genetic factors between the various strains of a species may differentially interact with the stimulating features of an enriched environmental and bring-about different enrichment-mediated changes compared to controls.

Another factor that is likely to affect conclusions quite drastically is the nature of the standard environment to which the effects of enrichment are being compared. Studies that use rats have often used isolated housing conditions in their standard housing animal group, whereas studies of mice tend to use social housing. Animals reared within standard housing in isolation are well documented to exhibit many differences in behaviour and neurophysiology compared to those raised in groups (Hall, 1998). For example, consequences of isolation include increased levels of anxiety (Wright et al., 1991), impaired spatial learning (Juraska et al., 1984;Lu et al., 2003b;Hellemans et al., 2004) and altered synaptic transmission and plasticity in various brain regions including the dentate gyrus and CA1 region of the hippocampus (Lu et al., 2003b;Bartesaghi, 2004). Thus the differences between enriched animals and those from isolated conditions may not always reflect consequences of enriched experience but may more actually characterise the negative effects of social impoverishment.

1.11.2 Improved Learning and Memory

Since the observations of Hebb, it has been repeatedly shown that animals housed in enriched environments can exhibit enhanced performances in a variety of hippocampal-dependent behavioural tests of learning and memory. Types of memory found to be improved after enrichment include: spatial reference memory (as tested in the Morris water maze), contextual fear conditioning and novel-object recognition memory (e.g. Paylor et al., 1992;Moser et al., 1994b;Kempermann et al., 1997;Kempermann et al., 1998b;Tees, 1999;Rampon et al., 2000b;Duffy et al., 2001;Williams et al., 2001;Faverjon et al., 2002;Frick and Fernandez, 2003;Need and Giese, 2003).

Rodents have also been noted to remain susceptible to the cognition-promoting effects of enriched experience throughout their lifetimes (e.g. Winocur, 1998;Soffie et al., 1999;Kempermann et al., 2002;Frick and Fernandez, 2003;Frick et al., 2003). The

persistence of its cognitive effects in conjunction with the now well documented findings that enriched animals exhibit fewer impairments and/or improved abilities to recover from physical, chemical and genetic lesions in the brain (e.g. Hockly et al., 2002;Johansson, 2003;Dahlqvist et al., 2004) has led to the proposal that enrichment should not be considered as enhancing brain function over a natural level but that it might more accurately be regarded as either reversing or compensating for potentially negative affects of standard housing conditions upon brain function (Van Praag et al., 2000;Wurbel, 2001).

1.11.3 Altered Gene and Protein Expression Levels

Numerous molecular changes have been found in the brains of enriched animals suggestive of a history of increased levels of neural activity and synaptic plasticity and thus support the general hypothesis that enhanced experience must drive such processes. For example, it has been found that exposing animals to enriched environments can alter the expression level of numerous genes and proteins that are involved in synaptic transmission and the modification of neuronal structure and synaptic efficacy (studies have focused primarily on the hippocampus and cortex). For example, Rampon et al. (2000) used high-density oligonucleotide microarrays (gene chips) to analyse gene expression in the cortex following exposure to enriched environments. They identified approximately 100 genes whose total expression levels within the cortex were altered by more than 1.5-fold after raising the animals in enriched conditions for either 2 or 14 days (Rampon et al., 2000a). Most of the genes encoded proteins involved in synaptic transmission, neuronal signalling or structural plasticity (Rampon et al., 2000a). Interestingly, this study also found that after only 3 or 6 hours of enrichment, genes with expression levels that were altered by at least 1.5-fold were mostly different from those identified after days of enrichment. Nearly 50% of the genes at these early time points encoded proteins involved in *de novo* protein synthesis suggesting that one of the first consequences of enriched experience is a priming of the molecular machinery that will enable the subsequent up-regulation of specific proteins and thus the function of the experience-sensitive neural systems.

Note, the lack of any change in the expression level of mRNA for a particular protein does not necessarily mean that its level of protein activity is not altered by enriched

experience as the level of mRNA translation may be increased without changes in transcription levels. Alternatively, changes in mRNA or protein levels that occur in a relatively small proportion of neurones may hold important functional significance but the changes may be too small to detect when whole brain homogenates are analysed. Thus, although gene-chip technology is useful for screening large numbers of potential transcripts that could undergo large overall changes, they may not reveal significant enrichment-mediated changes that occur in a more specifically restricted manner. To investigate the effects of enrichment upon the expression of specific molecules of interest, studies have used techniques such as immuno-staining or mRNA *in situ* hybridisation techniques in brain sections or other specific tissue preparations.

Some of the molecules found to be up-regulated by enrichment include proteins involved in synaptic vesicle trafficking and the presynaptic release of neurotransmitter, such as synatophysin, synaptobrevin and clathrin AP-2 (Rampon et al., 2000a;Nithianantharajah et al., 2004), and proteins involved in postsynaptic signalling cascades, including PKC, CaM and PSD-95 (Paylor et al., 1992;Rampon et al., 2000a;Nithianantharajah et al., 2004). Other molecules found to undergo large expression changes include those involved in the modulation of neuronal structure. This category of molecules includes neuronal adhesion molecules, like integrin-α4 and N-Cadherin (Rampon et al., 2000a), the neurotrophins BDNF and NGF (Falkenberg et al., 1992;Pham et al., 1999;Mohammed et al., 2002;Pollak et al., 2005) and various cytoskeletal molecules including dynactin and arc (Rampon et al., 2000a;Pinaud et al., 2001). Expression of the transcription factor CREB, whose activity has been identified to be required for both late-phase LTP and memory (Bourtchuladze et al., 1994), has also been identified to be up-regulated following enrichment (Williams et al., 2001).

Of particular interest for this thesis was the finding that the gene for CaM was up-regulated after enrichment whilst that for neurogranin (the postsynaptic CaM-binding protein that buffers free CaM at basal Ca^{2+} concentrations) was down-regulated (Rampon et al., 2000a). If such changes also correlate on the protein level they would implicate increased levels of CaM-dependent signalling and also therefore the activity levels of CaM-stimulated enzymes like CaMKII, adenylyl cyclases 1 and 8 and the phosphatase PP2B in the brains of enriched mice. As such molecules are all considered

to be implicitly involved in the bidirectional modification of synaptic strengths it again supports the use of environmental enrichment and CaMKII mutant mice to investigate experience-dependent synaptic plasticity.

Environmental enrichment has also been found to result in a 2.5-fold decrease in the cortical levels of the mRNA for the PKA regulatory subunit (Rampon et al., 2000a); this implicates an experience-dependent *increase* in PKA signalling despite no detectable changes in the mRNA levels of the catalytic subunit. As discussed in the previous sections PKA plays an important role in CaMKII-dependent LTP and long-term memory, again supporting the hypothesis that enrichment-mediated plasticity may trigger LTP-like phenomena to take place. Indeed, the overexpression of this regulatory subunit in transgenic mice was previously found to significantly impair the late-phase of LTP as well as long-term memory (Abel et al., 1997).

Increases in glutamate receptor protein levels have also been observed in the mouse brain following the exposure to enriched environments. One study assessed the levels of the GluR1 AMPA receptor subunit and the NR1, NR2A and NR2B NMDA receptor subunits in forebrain homogenates of enriched mice compared to levels in control mice from standard housing. Subunit expression levels were significantly increased for all except the NR1 subunit following 2 weeks of enrichment. These enriched mice also exhibited enhanced learning abilities compared to controls, thus correlating these molecular changes with enhanced cognitive abilities (Tang et al., 2001b). More recently, the effects of enrichment upon the mRNA levels for the GluR2 and GluR4 AMPA receptor subunits were also assessed in mice and were also found to be significantly increased compared to controls (Naka et al., 2005).

Other studies, in rats, have found that enrichment can result in a large increase in the surface expression of AMPA receptors in the rat hippocampus (assessed using ^{3}H-AMPA binding assays; Foster et al., 1996; Gagné et al., 1998). Moreover, the capacity for Ca^{2+} to up-regulate ^{3}H-AMPA binding was significantly decreased in the enriched animals (Gagne et al., 1998) whilst total levels of both AMPA receptor mRNA and protein were unaffected (Foster et al., 1996;Gagne et al., 1998). These finding together suggest that enrichment in these studies triggered the redistribution of AMPA receptors from intracellular sites into the membrane, again supporting the hypothesis that this

housing paradigm promotes experience-dependent synaptic plasticity in the hippocampus. The discrepancy between the above studies regarding unchanged AMPA receptor expression levels in rats (Foster et al., 1996;Gagne et al., 1998) compared to the increases seen in mice (Tang et al., 2001b) may characterise a species-specific difference in enrichment-mediated responses in the brain. Alternatively it may reflect the use of different control conditions in the rat and mouse studies; the rat studies using isolated control animals whilst the mice were raised in social housing.

1.11.4 Anatomical Changes in the Brain

Many anatomical changes have also been found to occur in the brains of enriched animals. In fact the ability of enriched housing and formal training paradigms (i.e. behavioural tests of learning and memory) to induce structural as well as biochemical changes in the brain were some of the first lines of evidence that supported the hypothesis that functional plasticity may exist in the brain (e.g. Krech et al., 1956;Krech et al., 1960;Diamond et al., 1964) – predating the first demonstration of activity-induced synaptic plasticity (i.e. Bliss and Lomo, 1973).

The early anatomical studies of environmental enrichment from the 1960's and 1970's primarily focused on regions of the cerebral cortex and revealed that housing rats in such conditions could lead to structural growth at several anatomical levels (reviewed in Rosenzweig and Bennett, 1996). Enriched experience was found to lead to increases in cortical thicknesses (Diamond et al., 1964) and weights (Bennett et al., 1969), as well as increases in the sizes of neuronal cell bodies and nuclei (Diamond et al., 1975) and the rate of glial cell proliferation (Altman and Das, 1964;Diamond et al., 1966;Szeligo and Leblond, 1977). Enriched-mediated increases were also found on the subcellular level, and included measures of dendritic branching (Holloway, Jr., 1966;Volkmar and Greenough, 1972;Greenough and Volkmar, 1973), dendritic spine and synapse densities (Globus et al., 1973;Bhide and Bedi, 1984) and size of synaptic contact areas (West and Greenough, 1972). These finding prompted the suggestion that enriched-experience can induce a substantial increase in the processing capacity of the cerebral cortex (Rosenzweig and Bennett, 1996).

Since the development of more advanced imaging techniques, as well as experimental

paradigms of synaptic plasticity, more direct lines of evidence that been published supporting the hypothesis that the structural plasticity of dendritic spines may accompany persistent forms of synaptic plasticity in the brain (e.g. Engert and Bonhoeffer, 1999;Toni et al., 1999) and that such changes may represent structural correlates memory formation (e.g. Lendvai et al., 2000;O'Malley et al., 2000;Knafo et al., 2001;Knafo et al., 2004; section 1.8). Indeed, such findings likely fuelled the more recent resurge of interest into the effects of enrichment upon spine number/density in brain regions like the neocortex and hippocampus, with enrichment providing a convenient paradigm to study experience-dependent plasticity in the brain.

Enriching the environment of adult rats for 3 months has been found to correlate with increases in spine densities in pyramidal cells of the parietal cortex, occipital and prefrontal cortices as well as in the medium spiny neurones of the nucleus accumbens compared to control rats from isolated standard housing (Johansson and Belichenko, 2002;Kolb et al., 2003a;Kolb et al., 2003b). Increases in dendritic arborisation and total length were also observed in some cortical regions (Kolb et al., 2003a). Kolb and colleagues also showed that the morphological effects of enrichment differed depending upon animal sex and age and cortical region, indicating that the effects of enrichment upon neuronal structure are not universal (Kolb et al., 2003a;Kolb et al., 2003b). For example, depending on the age of the animals, enrichment could cause either an increase or decrease in spine density in the parietal and visual cortices; significant deceases in spine densities were found if the rats were transferred to enriched conditions immediately after weaning (21 days old), whereas increases were observed when either young adults (4 months of age) or senescent rats (2 years old) were transferred to enriched living habitats (Kolb et al., 2003a). The observed decrease in spine densities in the juvenile rats contrasts however with the findings from a different group who found that after only four days of enrichment, spine densities were increased by 30% in the basilar dendrites of visual cortex pyramidal neurones in rats 30 days old compared to isolated controls (Wallace et al., 1992).

Enriched housing has also been found to cause a 30% increase in spine density in the medium-sized spiny neurones of the rat striatum (Comery et al., 1995). This group then went on to show that the density of multiple-headed spines on these striatal neurones

were disproportionately increased by enrichment, with dendritic segments from enriched rats exhibiting 60% more multiple-head spines than those of the individually caged animals (Comery et al., 1996). This finding suggests that experience-mediated spinogenesis may selectively increase the density of specific types of dendritic spines (Comery et al., 1996). Note, it was not shown in this study (nor in the studies mentioned above) whether the new spines also received synaptic innervation.

With respects to the hippocampus, it has been found that exposing rats to enriched environments for 4 hours a day for 14-18 days resulted in a significant increase in spine density on the basal dendrites of CA1 neurones compared to control groups housed in standard conditions either individually or in pairs (Moser et al., 1994b). No differences in spine densities existed between these 2 control groups and the total dendritic length and degree of branching within the basal dendrites were similar between enriched and control groups. In a second investigation, Moser and colleagues repeated this finding and also revealed that the increased spine densities were not spread evenly throughout the basal dendrites but were concentrated within a small subset of dendritic segments per cell (Moser et al., 1997). They extended the analysis to the apical dendrites of CA1 pyramidal neurones and found that the spine densities remained stable within these dendritic compartments (Moser et al., 1997). This, however, contrasts with the results from a study in mice, where CA1 apical dendrite spine densities were found to be increased after 2 months of daily enrichment (3 hours per day; Rampon et al., 2000b). This mouse study also showed, using electron-microscopy, that the density of axospinous non-perforated synapses within the stratum radium of the hippocampus was also increased with enrichment, suggesting that the additional spines are innervated and potentially functional.

This discrepancy may reflect differential responses between rats and mice or it may also be a consequence of differences in the enriching paradigms used. In order to provoke exploration Moser et al. (1994 and 1997) deprived their rats of food and water for 19 hours before they were placed in the enriched environment for 4 hours daily where they had to hunt for their hidden food and water bowls. The complex environment was a five cubic meter cage with numerous levels and interconnected platforms that were covered with various materials and distributed objects that were changed on each day of

enrichment. In contrast, the mice of Rampon et al. (2000b) were not food and water deprived; thus their exploratory behaviour in the enriched cages may have held different motivational significance. The mice were also exposed to enrichment for 2 months whilst the rats received only 2-3 weeks of enrichment training. On a note of similarity, both studies found that their enrichment paradigms resulted in enhanced cognitive abilities in the enriched animals. Moser et al. (1994) found that enriched animals showed improved spatial learning abilities in the Morris water maze and Rampon et al. observed improved performances in tests of non-spatial forms of hippocampal-dependent memory.

Environmental enrichment has also been found to enhance the number of new neurones within the hippocampal formation. Neurogenesis in the adult brain is a phenomenon that is restricted to the dentate gyrus of the hippocampus and the olfactory bulb (Altman, 1962;Luskin, 1993). Thousands of new neurones are produced daily in these regions and throughout the lifetime of an animal (Gage et al., 1998). In the dentate gyrus these new neurones are produced from progenitor cells located within the subgranular zone; new daughter cells then migrate into the granule cell layer where they can become functionally integrated into existing circuits, receiving synapses and extending axonal connections to CA3 pyramidal cells (Van Praag et al., 2002). The majority of newly generated cells degenerate within a few weeks after cell division (Gould et al., 1999;Biebl et al., 2000); however, environmental enrichment has been found to enhance the survival of these new granule cells (Kempermann et al., 1997;Kempermann et al., 1998a;Kempermann et al., 1998b;Nilsson et al., 1999). The neural consequences of enrichment are therefore proposed to promote the expression of specific survival-factors in the brain; candidate molecules are likely to include various growth factors, like BDNF and NGF whose expression levels are increased with enrichment (Falkenberg et al., 1992;Mohammed et al., 2002).

Increased levels of voluntary exercise in rodents (provided for in the form of a running wheel) have also been found to enhance the survival of newly developed cells in the dentate gyrus, even in the absence of other enriching stimuli (Van Praag et al., 1999b). Thus the increased physical activity in the enriched environments is one factor that is likely to contribute towards the biochemical changes that promote the survival of new

granule cells. Learning and electrical stimulation of the dentate gyrus have also both been found to promote neurogenesis in the dentate gyrus (Gould et al., 1999;Derrick et al., 2000), thus the effects of exercise might simply arise due to increased levels of neuronal input into the hippocampal formation. Indeed motor activity is known to correlate with increased hippocampal theta rhythm and the discharge frequencies of running-sensitive hippocampal pyramidal cells and interneurones have even been seen to increase with increased wheel running velocity in rats (Czurko et al., 1999).

Running, but not enrichment, has also been found to increase the rates of cell proliferation in the dentate gyrus (Van Praag et al., 1999b); thus it also remains possible that the mechanisms by which enrichment and running increase cell numbers may differ (Van Praag et al., 2000). Interestingly, neither enrichment nor running were found to increase the number of newly generated cells in the olfactory bulb, suggesting that the interaction between the environment and neurogenesis is a phenomenon specific to the dentate gyrus (Brown et al., 2003).

Both running and enrichment have, however, been associated with improved learning abilities in rats (e.g. Kempermann et al., 1998b;Van Praag et al., 1999a). A recent study addressed the question of whether newly generated neurones in the dentate gyrus were themselves involved in the expression of enhanced long-term memory following environmental enrichment (Bruel-Jungerman et al., 2005). To address this, rats received daily injections of an anti-mitotic agent throughout the 14 day enrichment period and the effects upon memory and neurogenesis were compared with untreated and saline injected controls. In accordance with previous studies, enrichment was found to lead to increased neurogenesis and improved long-term recognition memory in both the untreated and saline injected rats but neurogenesis and improved learning were both prevented in the rats that received the anti-mitotic agent, thus supporting the hypothesis that newly born granule cells in the dentate gyrus may directly participate in enrichment-stimulated enhanced cognitive function (Bruel-Jungerman et al., 2005).

1.11.5 Effects of Enrichment upon Synaptic Transmission and Plasticity

The properties of LTP in hippocampal slices from enriched animals have also been found to be sensitive to enrichment. For instance, the magnitude of LTP in CA1 was

found to be greater in slices from enriched mice compared to those from control mice (Duffy et al., 2001). The enhanced levels of LTP found in this study did not correlate with altered biophysical properties of the CA1 neurones or with altered baseline responses, but they were associated with an increase in the dependence of LTP upon PKA activity (Duffy et al., 2001). This finding is supported by the noted observation of Rampon and colleagues (2000a) that enrichment reduces the mRNA levels of the PKA inhibitory subunit in mice suggesting enhanced PKA function in enriched brains.

In contrast, a study in rats has found that the magnitude of CA1 LTP in hippocampal slices is not altered in enriched animals compared to non-enriched controls; however the baseline measures of synaptic strength were increased in the enriched animals and further investigation attributed these changes to enhanced postsynaptic properties while presynaptic properties remained unaffected (Foster and Dumas, 2001). This finding was also consistent with two earlier studies in rats, where enrichment was found to increase the AMPA receptor binding in the rat hippocampus (Foster et al., 1996;Gagne et al., 1998), while the magnitude of Ca^{2+}-stimulated increases in surface expression was decreased compared to controls (Gagne et al., 1998).

The differential effects of enrichment upon CA1 LTP may again be either be species related, or reflect differences in enrichment procedures. The mice of Duffy et al. (2001) were permanently housed in an enriched cage that was not altered during the 2 month enrichment period and the control mice were housed in small groups within standard conditions. On the other hand, the rats of Foster et al. (1996) were housed in larger cages containing several objects and were additionally exposed to one of several novel enriched environments for up to 6 hours a day for 25-32 consecutive days while control rats were kept in isolated cages. Thus the differential effects of the enrichment upon LTP in these studies might: 1) reflect the effects of isolation housing in the rat study by Foster et al., 2) be due to species differences or 3) be related to the different exposure levels to novelty that the two enrichment paradigms offer. Nevertheless, both experiments show that experience in the form of enriched housing environments can affect the properties of synaptic physiology in the hippocampus and again supports the rational for using environmental enrichment in this thesis.

Enrichment has also been shown to affect LTP in the dentate gyrus. Reduced magnitudes of medial perforant path LTP were found in enriched rat brain slices compared to those assessed in slices from rats housed in individual cages (Foster et al., 1996). This reduction in the magnitude of LTP correlated with enhanced baseline synaptic efficacies assessed before LTP induction (characterised by the slope of the evoked EPSP) and suggest that the enriched experience itself had resulted in the potentiation of perforant path synapses (Foster et al., 1996). Furthermore, these experience-dependent effects could be blocked in rats that received the NMDA receptor antagonist AP5, administered to the lateral ventricle *in vivo* via implanted cannulae throughout the 2 week enrichment period (Foster et al., 2000). This provides evidence that the enrichment-mediated modifications of perforant path synaptic function were indeed dependent upon NMDA receptor-mediated synaptic activity. It also suggests that the neural mechanisms underlying this plasticity may interact with the mechanisms that underlie LTP (Foster et al., 2000); thus supporting the proposal that phenomena similar to LTP may exist *in vivo* and are recruited during experience-triggered information processing and storage.

CHAPTER 2
Methods

2.1 Experimental Animals

The subjects were male αCaMKII^{T286A} homozygote mutant mice (Giese et al., 1998) and their wild-type littermates. The animals used were between six and eight weeks of age. All experiments and data analyses were performed blind to genotype.

During the first year of experimentation, animals used were derived from heterozygous αCaMKII^{T286A} breeding pairs that had been previously back-crossed into the C57BL/6 inbred mouse strain five times (Giese et al., 1998). These animals were used for the *in situ* hybridization and immunocytochemistry experiments and the initial confocal microscopy imaging studies of hippocampal CA1 pyramidal cells.

Due to the low breeding efficacy of this inbred strain, all subsequent animals were bred from intercrosses of the F1 heterozygote offspring (cousin matings) generated by crossing αCaMKII^{T286A} homozygote mutant males with inbred 129 S2/SV wild-type females (Harland Animal Suppliers, UK). All breeding pairs were housed in standard mouse laboratory cages (see below) and the weaning of litters was performed at postnatal day 21 (P21).

2.2 Animal Housing Conditions

Male mice were weaned into either standard or enriched housing conditions. All animals were exposed to a 12 hour light-dark cycle with food and water provided *ad libitum* from the cage lid. The standard housing condition consisted of a standard plexiglass mouse laboratory cage (dimensions*: 35 x 20 x 14 cm), lined with sawdust

* length x width x height

shavings and a small amount of tissue paper was provided as bedding material. Mice were housed in groups of 3 to 5.

The enriched housing condition consisted of a large plexiglass laboratory cage (dimensions: 35 x 43 x 21 cm), containing a medley of bedding materials (e.g. sawdust, shredded paper, various grades of wood chippings, rodent bedding wool, paper bags and large sheets of paper roll) and novel objects. Typical objects included: various purpose-built rodent houses, tubes – of different materials and sizes, running wheels, ladders, see-saws, ropes, cardboard test-tube racks and many other items (figure 2.1). Objects were frequently suspended from the lid of the enrichment cage (something that mice always seemed to particularly enjoy!). The cage contents were changed on a daily basis ($\geq$ 5 times per week) so that the combination and arrangement of items was never repeated. Mice in the enriched housing condition were housed in groups of 5-15.

2.3 Genotyping

2.3.1 Preparation of Genomic DNA from Mouse Tail

DNA, for genotyping, was obtained from tail biopsies taken on the day of weaning. Each biopsy (a tail snip, approximately 5mm long) was placed into a separate 1.5 ml Eppendorf tube labelled with the specific mouse identification code. 0.5 ml tail lysis buffer plus 0.5 mg proteinase K (see table 2.1) was added to each tail biopsy; the biopsies were incubated at 55°C overnight. Each digested biopsy was then vortexed for approximately 1 minute in order to maximise the release of genomic DNA from the digested tail tissue into solution. Digests were spun in a bench-top centrifuge (Biofuge, Heraeus Instruments, U.S.A.) at full speed (13000 rpm) for 10 minutes and the DNA containing supernatants decanted into fresh Eppendorf tubes. 500 µl isopropanol (100%) was added and each tube inverted several times until a white cloudy precipitate of DNA appeared. The DNA was carefully picked out using a clean Gilson pipette tip and transferred to a new Eppendorf tube containing 500 µl ethanol (70%). Tubes were again spun for 1 minute, the ethanol aspirated away and the DNA pellets left to dry at room temperature for 30 minutes. DNA was resuspended in 50 µl Tris buffer, pH 8.5 (10 mM), by incubation overnight at 55 °C.

Figure 2.1 The Enriched Housing Environment.

A) This photograph shows a typical example of the enriched housing environment. At least 5 days a week the mice were transferred into a freshly prepared cage containing a new combination of objects and bedding materials that varied in material, size and number. Objects, such as cardboard and plastic tubes, ropes and cardboard houses were also suspended from the wire lid of the cage.

B) The mice can be seen to be actively exploring their new habitats and to be making use of the exercise wheels provided (a particularly popular activity with the mice!).

A) An example of enriched housing conditions (with lid removed)

B) Mice exploring their new environments and undertaking voluntary exercise.

Table 2.1 Composition of Tail Lysis Buffer.

Tail lysis buffer was made up as a 1x stock solution and kept at 4 °C. When required, the calculated volume of tail lysis buffer was decanted and Proteinase K added to give an end concentration of 1 mg/ml. Proteinase K stock (50 mg/ml, dissolved in 100 mM Tris-HCl pH 8.5) was stored at -20 °C. All reagents were from VWR international, U.K.

Chemicals	Tail Lysis Buffer
Tris-HCl pH 8.5	100 mM
EDTA*	5 mM
NaCl	0.2 mM
SDS**	0.2 %
Made up in MilliQ-purified water Plus 0.5 mg Proteiase K per tail	

* Ethylenediaminetetra-acetic acid disodium salt

** Sodium dodecyl sulphate

2.3.2 PCR Analysis

To produce the point mutation in the αCaMKII allele, Giese et al (1998) used a gene-targeting strategy that utilised a replacement vector containing the CaMKII gene with the T286A point mutation and a neo gene flanked by loxP sites (the Pointlox procedure). The presence of wild-type and/or mutant αCaMKII alleles within each mouse's genomic DNA sample was determined using polymerase chain reaction (PCR) analysis (GeneAmp PCR System, Applied Biosystems, CA, U.S.A). PCR primers were specifically designed to amplify out the region of DNA that, only in the mutant alleles, would contain the residual Lox P sequence (34 base pairs (b.p.) in length). Thus, the presence of wild-type and mutant alleles was easily distinguishable by separation and visualisation of the PCR products by agarose gel electrophoresis (BioRad, CA, U.S.A).

Since there is a logarithmic relationship between the size of a DNA fragment and the distance it migrates on an agarose gel, the two PCR products (i.e. the shorter wild-type and longer mutant transcript) were identified by their relative distances travelled. Verification that the PCR products were of the expected sizes was achieved by running a 1 Kb DNA molecular weight marker (1 Kb ladder) on every gel.

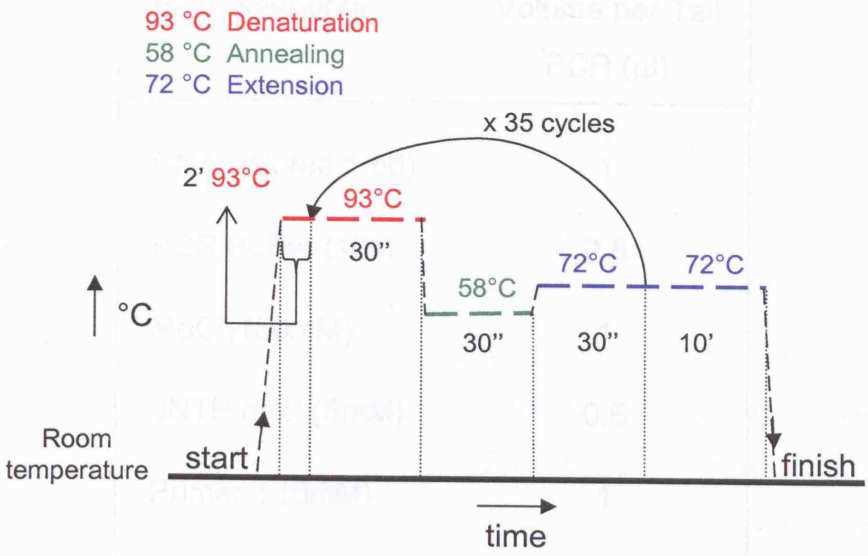
Approximately 5-10 μg of DNA (1 μl of a 1 in 50 dilution of mouse DNA sample) was used per PCR tube (i.e. per mouse). The PCR reagents and recipe followed are shown in table 2.2; the PCR protocol is schematically represented in figure 2.2. The nucleotide sequences of the two PCR primers used were as follows:

Primer 1 - TGTACCAGCAGATCAAAGC

Primer 2 - ATCACTAGCACCATGTGGTC

Within the electrophoresis tank, the agarose gel (12% agarose solution dissolved in TAE buffer – 0.04 M tris-acetate and 0.001 M EDTA – plus 2.5 μl/100 ml EtBr) was immersed in TAE buffer. 12 μl of each PCR product, plus 2 μl loading buffer (bromophenol blue indicator grains dissolved in 100% glycerol), was loaded into the agarose gel; all reagents were obtained from VWR International (U.K.). The PCR samples, plus one lane containing the 1 Kb ladder, were run for approximately 30 minutes at 100 mV. The separated EtBr-collated DNA bands were visualised using a UV trans-illuminator and camera (AlphaImager 2000, Alpha Innotech Corp., CA, U.S.A.); a digital image of the gel was printed as a permanent record of the mouse genotypes.

Figure 2.2 Schematic Diagram of the PCR Protocol



The PCR cycle was initiated by heating the PCR tubes to 93°C to promote the denaturing of mouse genomic DNA. The first round of denaturing lasted for 2 minutes 30 seconds. The tubes were then rapidly cooled to 58°C for 30 seconds – the temperature at which the two oligonucleotides PCR primers could anneal to the complementary strands of DNA. Extension of the PCR primers was catalysed by Taq polymerase and performed at 72°C for 30 seconds. 35 cycles of this sequence of temperatures was performed. After the final cycle, with an additional 10 minutes extension time, the tubes were cooled and maintained at room temperature. The size of the PCR products for each DNA sample, and thus the genotype of each mouse, were identified by running the contents of the PCR tube on an electrophoresis gel.

Table 2.2 PCR Reagents and Recipe.

Taq polymerase and primers 1 and 2 were obtained from Invitrogen (U.K.) and Invitrogen Custom Primers (U.K.) respectively. The dNTPs came from Amersham (U.K.) and the PCR buffer and $MgCl_2$ from VWR International (U.K.).

PCR Reagents	Volume per Tail PCR (μl)
DNA (diluted 1:50)	1
PCR Buffer (10x)	2.5
$MgCl_2$ (50mM)	1
dNTP mix* (5mM)	0.5
Primer 1 (5mM)	1
Primer 2 (5mM)	1
Taq Polymerase	0.125
Water	17.875

*deoxynucleotides: dATP, dCTP, dGTP and dTTP (each at 5mM).

2.4 In Situ Hybridization

The *in situ* hybridization of radiolabelled oligonucleotide probes against αCaMKII messenger ribonucleic acid (mRNA) in mouse brain sections was performed in collaboration with Dr. Florentina Soto at the Max-Planck-Institut für Experimentelle Medizin, Göttingen, Germany. All tissue preparation was carried out at U.C.L and the tissue subsequently transported to Göttingen for hybridization and X-ray film exposure.

A potential problem in mRNA hybridization studies is the degradation of signal mRNA

due to contamination of the preparation with environmental RNases. To minimise this problem, all heat resistant equipment was baked at 200°C for a minimum of 8 hours, and the distilled water (used for the preparation of solutions and the washing of equipment) was treated with 0.05% diethylpyrocarbonate (DEPC; Sigma) before twice autoclaving for 20 minutes. No apparatus was touched without wearing gloves, reducing the risk of re-contamination of the preparation with RNase.

2.4.1 Tissue Preparation and Fixation

P1, P2, P3, P4, P8 and adult (P38) mice were decapitated, their brains carefully removed and rapidly frozen on dry ice. With respects to the pre-weaned mice, entire litters were taken and all brains sectioned, fixed, dehydrated and stored at 4°C in absolute ethanol, on the same day as dissection. Tail biopsies from each pup were subsequently genotyped to identify homozygote wild-type and mutant animals. Adult mice were genotyped post weaning and only wild-type and mutant animals selected for sectioning. 9 entire litters (aged P1-P8) plus 4 adult brains were sectioned – a total of 53 animals. The P1-P8 litters gave: 14 wild-types, 9 mutants and 29 heterozygotes. 4 adult mice were sectioned: 2 wild-types and 2 mutants. Wild-types animals were obtained at all ages, but, unfortunately, no homozygote mutant animals were obtained for the litters sliced at ages P2 and P8.

Cryostat (Zeiss) parasagittal brain sections (15 μm) were thaw-mounted onto silan-coated slides (3'-aminopropyltriethoxysilan; Sigma). From each brain, sagittal sections were cut, starting in line with the lateral edge of the olfactory bulb and finishing just before the inter-hemisphere fissure was reached. Sections were then fixed with 4% paraformaldehyde (dissolved in phosphate buffered saline, PBS: 137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4.7H_2O$; pH 7.4) at 4°C for 25 minutes, washed for 3 x 10 minutes in PBS and then dehydrated via an ethanol chain (5 minutes in each of 50, 75, 90 and 2 x 100%). Sections were stored in 100% ethanol until further use.

For transportation to Germany, the selected slides were air-dried overnight and stacked into slide boxes (washed thoroughly with DEPC-treated water) which were then packed into dry-ice for transport. On arrival, the slides were stored at -80°C.

2.4.2 *Preparation of Oligonucleotide Probes*

Two anti-sense oligonucleotide probes were generated (Metabion AG, Germany; designed by Dr. F Soto) complementary to mRNA nucleotides 80-129 (Probe A) and 1074-1120 (Probe B) of the murine αCaMKII sequence. A control sense probe corresponding to mRNA nucleotides 80-129 (i.e. complementary to the Probe A sequence), was also used. Sequences were checked by a BLAST® search (http://www.ncbi.nlm.nih.gov) against the NR database (comprises all non-redundant GenBank, EMBL, DDBJ and PDB sequences) for specificity and no other matches were found.

The probe sequences were as follows:

Probe A:
TGGCACTGGGCAGGCAGGCGAGGCTTTAGGCTGGGAACTAGGGCTGGGAT

Probe B:
GATTCCTTCACACCATCGTTCTTCTTGTTTCCTCCGCTCTTCCCTCC

Sense probe:
ATCCCAGCCCTAGTTCCCAGCCTAAAGCCTCGCCTGCCTGCCCAGTGCCA

Oligonucleotide probes were 3'-end labelled with ^{35}S-ATP (DuPont/NEN; 1200 Ci/mmol). Each probe (approximately 7.5 ng) was incubated for 8 minutes, at 37°C, in the presence of terminal deoxynucleotidyl transferase (TdT), TdT-buffer, $CoCl_2$ (5 mM; TdT co-factor; all Boehringer Mannheim, Germany) and ^{35}S-ATP. The reaction was terminated by the addition of STOP-buffer (0.14 M NaCl, 0.02 M Tris-HCl pH 7.5, 0.025 M EDTA and 0.1% SDS). Unlabelled probes were removed using Bio-Spin 6 centrifugation columns (Bio-Rad, CA, U.S.A.); the reaction mix was transferred to the columns and spun for 30 seconds at 3500 rpm. Labelled probes were stored in 1 M DTT (dithiothreitol) at -20°C.

2.4.3 Hybridization of Probes

The slides were removed from the -80°C store and thawed at room temperature. Slides were first fully hydrated by submersion into DEPC-treated purified water and then re-dehydrated via an ethanol chain (5 minutes in each of 25, 50, 75, 90 and 2 x 100%). Selected slides were removed from the 100% ethanol, air dried and hybridized overnight at 42°C in 100 µl hybridization buffer containing 4 × 105 cpm (~ 2-5 pg/µl) of radio-labelled probe plus (control slides) or minus (experimental slides) 500-fold excess unlabelled probe (hybridization buffer: 50% formamide, 10% dextran sulphate, 50 mM DTT, 0.3 M NaCl, 30 mM Tris-HCl, 4 mM EDTA, 1x Denhardt's solution, 0.5 mg/ml denatured salmon sperm DNA, and 0.5 mg/ml polyadenylic acid). After hybridization, slides were washed twice at room temperature for 20 minutes in 1x SSC (0.15 M NaCl plus 0.015 M Na citrate adjusted to pH 7 using HCl) containing 50 mM β-mercapotoethanol. This was followed with a 30 minute incubation in 1x SSC at 57°C, then 5 minutes incubation in 1x SSC and 5 minutes in 0.1x SSC both at room temperature. Specimens were subsequently dehydrated (5 minutes in each of 50, 75, 90 and 2 x 100%), air-dried and exposed to Kodak Biomax X-ray film (Eastman Kodak, Rochester, NY, U.S.A.) for 21 days.

Films were imaged with a monochrome CCD camera and digitised using M5+ software (Imaging Research Inc., courtesy of Professor C. Yeo, Department of Anatomy, U.C.L.) Using M5+, the original grey-scale autoradiograph digital images were converted to a linear pseudo-colour scale. The colour scale corresponds to increasing optical densities and thus enables the images to highlight the relative signal intensities in different brain regions.

2.5 Immunocytochemistry

To determine the localisation of αCaMKII protein in P1, P4, P8 and adult (P35) mouse brains, microtome sections were first processed for immunocytochemical detection using monoclonal anti-mouse αCaMKII antibodies. Biotinylated secondary antibodies were then added and in turn detected using the avidin-biotin enzyme complex and visualised with nickel enhanced diaminobenzidine (DAB) staining. DAB is the substrate for the conjugated peroxidase enzyme and produces a grey-black reaction product in the

presence of nickel.

Animals were deeply anaesthetised using sodium pentobarbital (P1-P8, 10 µl; adults, 50 µl), and transcardially perfused with 0.9% saline containing heparin, followed by 4% paraformaldehyde (PFA) dissolved in 0.1 M phosphate buffer, pH 7.4 (PB: 4 parts 0.1 M Na_2HPO_4 plus 1 part 0.1 M NaH_2PO_4,). Brains were removed and post-fixed (4% PFA, plus 30% sucrose – a cryo-protector) for 1 hour at 4°C and then transferred into a 0.1 M PB solution containing 5% sucrose and 0.02% sodium azide (a preservative) and stored at 4°C until sectioning. Tail biopsies from the P1, P4 and P8 animals were subsequently genotyped to identify homozygote wild-type and mutant animals; heterozygote brains were discarded.

Parasagittal whole brain slices (50 µm) were taken using a freezing microtome. Sections were individually removed using a fine paintbrush and serially placed in wells containing 0.1M PB, 5% Sucrose and 0.02% sodium azide. Four to six free-floating microtome brain sections of each genotype and at each age were placed into separate wells that defined the experimental and control groups. The wells contained nets for easy transfer between the various reagents and the multiple washes. All incubations and washes, unless stated, were made at room temperature on a jigger.

After 3 x 10 minute washes in 0.1 M PB (containing 0.3% Triton X-100 to permeablise the cell membranes) all sections were incubated for 30 minutes in 0.1 M PB containing 0.6% hydrogen peroxide (to block any endogenous peroxidase activity that would produce a reaction product from the enzyme substrate, DAB, alone). Sections were then transferred into 0.1 M PB containing 3% normal goat serum (to block non specific absorption of the primary antibody into tissue elements), 0.4% Triton X-100 and 0.02% sodium azide (PBB, phosphate buffered 'block') and incubated for 1 hour. For incubation in the primary antibody, sections were transferred into fresh PBB containing monoclonal anti-mouse CaMKII antibodies (1:1000) and incubated overnight at 4°C. Control sections underwent the same treatments, only without the primary antibody.

After 6 x 10 minute washes in 0.1 M PB, sections were incubated for 1 hour in the biotinylated secondary antibody – horse anti-mouse IgG (1:500 in PBB). After 3 x 10

minute washes in 0.1 M PB, sections were incubated for 1 hour in the avidin-biotin enzyme complex (ABC; 1:1000 in 0.1 M PB) that had been pre-mixed 30 minutes beforehand. After further washes (3 x 10 minutes in 0.1 M PB), the ABC-bound biotinylated secondary antibodies were stained by adding DAB – the substrate for the enzyme. The staining procedure entailed transferring the sections into 0.15 M tris-HCL, pH 7.4, containing 0.25 mg/ml DAB, 2 mg/ml nickel sulphate and 0.003% hydrogen peroxidase. The colour reaction was terminated by transferring the sections into distilled water. Control and experimental sections underwent identical staining treatments. Sections were washed in distilled water for 5 minutes and then mounted onto gelatinised slides and left to dry overnight. Slides were subsequently dehydrated through increasing alcohol concentrations (1 x 2 minutes in distilled water, followed by 2 x 2 minutes in each of 70%, 95% and absolute ethanol) and finally received 2 x 2 minute washes in Histoclear. Slides were cover-slipped using DPX as mountant.

Monoclonal anti-mouse CaMKII antibodies were obtained from Chemicon Temecula (CA, U.S.A.). The biotinylated horse anti-mouse IgG and the reagent kits for the avidin-biotin enzyme complex and DAB staining were obtained from Vector Laboratories Inc. (CA, U.S.A.).

Photomicrographs of control and αCaMKII stained sections were grabbed through a charge-coupled device (CCD) camera (JVC, London, U.K.) attached to a microscope and computerised on a Power G3 Macintosh using "Vision Explorer" software (Alliance Vision, Mirmande, France).

2.6 Electrophysiology

All the electrophysiological recordings were made in transverse slices of mouse hippocampus, obtained from 6-8 week old homozygote wild-type and mutant male animals with a mixed C57black6J / 129S2/SV background (see section 2.1).

2.6.1 The Acute Hippocampal Slice Preparation

Animals were killed by decapitation, abiding by the UK Home Office guidelines, and the head immediately submerged into a dissecting dish filled with ice-cold slicing

solution (see table 2.3 for slicing solution composition). Whilst submerged in the cold solution, the brain (minus the cerebellum) was quickly removed, hemi-sected and transferred to a beaker of the ice-cold slicing solution where it was left to chill for up to five minutes before further dissection. A second tail biopsy was taken from the deceased subject and genotyping performed again (at a later date) to reconfirm the genotype.

The slicing solution was prepared as a modified Krebs solution within which the partial replacement of Na^+ with sucrose, the increased Mg^{2+} and reduced Ca^{2+} content all aimed to reduce neuronal excitability and therefore reduce transmitter release in order to minimise the extent of cell death induced via glutamate excitotoxicity. Glutamate receptor antagonists were also added to the slicing solution for the same purpose.

The chilled hemispheres were gently transferred to a Petri dish also filled with ice-cold slicing solution and a segment from the top of each hemisphere cut away using a razor blade. The cut was made at an angle of approximately 105 degrees from the midline surface. The cut surface of each hemisphere was stuck down to the stage of a slicing chamber using cyanoacrylate glue. The slicing chamber contained frozen slicing solution up to the level of the stage and with the hemispheres glues in place was subsequently filled with ice-cold slicing solution. Whilst visualising the hemispheres under a dissecting microscope, 350 μm thick brain slices were cut using a vibrating tissue slicer with either a ceramic or carbon steel blade (Vibra-slice and Integra-slice, Campden Instruments, Loughborough, UK). Each hippocampal slice was dissected free from the brain slice and carefully transferred into an incubating chamber. All transport of hippocampal slices was carried out using a cut and fire-polished glass Pasteur pipette (tip diameter approximately 4 mm).

2.6.2 Hippocampal Slice Maintenance

The incubating chamber consisted of a 100 ml glass beaker containing a submerged platform upon which the hippocampal slices were placed. The platform was formed from a thin piece of cotton fabric pulled straight over a plastic ring and secured by wedging a second ring over the top. The incubating chamber solution was continuously bubbled with 95% O_2 / 5% CO_2; the chamber was warmed to and maintained at 34°C

using a water bath.

After all dissected hippocampal slices had been placed into the incubation chamber, the slices were transferred to a second incubating chamber containing fresh bubbled slicing solution also warmed to 34°C; this was found to improve the health of the brain slices.

Following a further 10 minutes incubation, the slicing solution was slowing replaced with bubbled Krebs solution also warmed to 34°C. The solution change-over was carried out for 25 minutes using a peristaltic pump that simultaneously pumped the fresh Krebs in whilst removing the mixed Krebs/slicing solution at the same rate (14 ml/minute). Once the solution transfer was complete, the incubation chamber with its gas perfusion system was removed from the water bath and allowed to equilibrate to room temperature. The slices remained undisturbed for at least 1 hour before being used for either electrophysiological recordings or for filling with fluorescent dyes.

2.6.3 Perfusion and Visualisation of the Slice Preparation

Individual slices were transferred to the glass bottomed recording chamber of an upright microscope (BX50WI, Olympus, Japan). Within the chamber, the slice was held in place by a lattice of 5 parallel nylon threads fixed to a U-shaped piece of flattened platinum wire. The recording chamber (volume ~ 1 ml) was continuously perfused with fresh Krebs solution (flow rate $\geq$ 1 ml/minute). The reservoir of Krebs was continuously bubbled with 95% O_2 / 5% CO_2, and was delivered to the recording chamber via gravity flow through a system of silicone tubing. A fine glass tube, connected via silicone tubing to a suction pump (HY-FLO, Medcalf Bros. Ltd., Herts, UK), constantly removed solution and ensured that the total volume of solution in the chamber remained constant. All drugs used during experiments were bath applied – added to the reservoir of Krebs solution before their delivery to the recording chamber.

The microscope was connected to an infrared CCD video camera (Hitachi Denshi Ltd., Japan) and the slices viewed on a monitor (Hitachi Denshi Ltd., Japan). Either a 40x or a 60x water immersion objective was used in combination with infrared differential interference contrast microscopy to visually identify the hippocampal CA1 pyramidal neurones and for the visual guidance and position monitoring of the recording and

stimulating microelectrodes.

The microscope and microelectrode manipulators were all mounted upon a vibration isolation air-table (Newport Corporation, CA, U.S.A.) to reduce mechanical disturbance of the preparation. The table and its load were positioned inside a Faraday cage to minimise externally derived electrical noise.

2.6.4 *Whole-Cell Voltage-Clamp Recordings*

Thick-walled borosilicate glass recording microelectrodes (World Precision Instruments 1B150F-3) were pulled on a two-stage vertical electrode puller (Model PP830, Narishige, Japan) to give a tip resistance of 4 – 5 MΩ. Electrodes were filled with a CsCl-based internal solution (for composition see table 2.4) using a MicroFil (World Precision instruments FL, U.S.A.) and inserted into the electrode-holder which contained a silver wire coated with AgCl – produced by previously immersing the silver wire in bleach for approximately 2 hours. The electrode holder was attached to the head-stage of an Axopatch 1D patch-clamp amplifier (Axon Instruments, CA, U.S.A.), and mounted on a micromanipulator (Märzhäuser, Wetzlar, Germany). The AgCl-coated silver wire formed the electrical interface between the ionic environment of the brain slice preparation and the electronics of the patch-clamp amplifier. The electrical circuit was completed by submerging the ground electrode, which consisted of a silver wire tipped with an AgCl pellet, into the Krebs solution within the recording chamber.

Silicone tubing, connected to the internal space of the electrode holder, allowed both positive and negative pressure to be applied to the interior of the recording electrode. This was achieved by blowing or sucking air gently through the tube. Once the tip of a recording electrode was submerged into the solution of the recording chamber, any potential difference between the recording and ground electrode was offset to zero. The electrode was visually guided towards the cell of interest whilst maintaining positive pressure to prevent the tip of the electrode from becoming blocked. This enabled neurones that lay just below the surface of the slice to be patched as this pressure can blow away any obscuring tissue.

The resistance of the electrode was continually monitored throughout the patching

procedure by applying a 5 mV test pulse (40 ms duration) every 100 ms. Contact between the electrode and the cell soma was detected visually and electrically – by observing an increase in electrode resistance. At this point the positive pressure was relieved from the electrode and gentle suction applied. The holding potential of the recording electrode was then set to -70 mV. A successful 'cell-attached' patch configuration was achieved by the formation of a high resistance seal ($\geq$ 1 GΩ) between the lipid of the cell membrane and the glass of the microelectrode.

Once in the cell-attached configuration, the pipette capacitance artefact on the test-pulse current response was cancelled (as much as possible) using the specific pipette capacitance compensation circuitry of the patch-clamp amplifier. Gentle pulses of suction were then applied to the recording electrode until the plasma membrane beneath the electrode tip was ruptured to create the 'whole-cell' configuration.

For all whole-cell voltage-clamp recordings, the holding potential was set to -70 mV. Error in the command potential due to 'series resistance' (resistance between the interior of the microelectrode and the cell soma) was left uncompensated, although a measure of it monitored regularly to assess its stability (see below). It was assessed that the disadvantages associated with series resistance compensation (i.e. the risk of circuit instability and 'ringing' at high compensation levels and increased electrical noise) outweighed any benefit gained by compensating for the error in holding potential. However, the magnitude of the *error* in the measured amplitude of a current flowing across the cell membrane caused by the series resistance is proportional to the actual magnitude of that current; thus, the benefits achieved from compensation were predicted to be minimal since the amplitudes of the synaptic currents being recorded were very small (4-10pA).

To assess the stability of recording conditions throughout the course of an experiment, in particular changes in series resistance, the total access resistance of the whole-cell configuration was regularly assessed by injecting 5 mV test-pulses. The amplitude of the current response to the injection was measured and the total access resistance calculated using Ohm's Law. Series resistance is one component of the total access resistance and is the most likely one to change and affect recording conditions.

Recordings were rejected if the peak amplitude of the current in response to the pulse altered by more than 25%. Test-pulses were performed after every evoked EPSC and approximately every 2-3 minutes in the miniature EPSC experiments.

A CsCl based intracellular solution was used in the patch electrode. Caesium ions are able to block potassium ion channels and thus increase the membrane resistance. This, in turn, increases the proportion of the holding voltage that is held across the membrane and decreases the proportion that is lost across the access resistances of the recording. This helped to reduce problems of imperfect space-clamp.

All recording were performed at room temperature (21-24°C).

2.6.5 Data Acquisition

Synaptic currents, detected in the voltage-clamp mode of the patch-clamp amplifier, were recorded at a bandwidth of 10 kHz (4-pole Bessel filter). A continuous recording of the membrane current of each individual experiment was digitised (CED1401, CED, Cambridge, UK) at 32 kHz, and stored onto DAT tape (Model VDAT2, Vetron technology Inc., PA, U.S.A.).

Recordings of the evoked synaptic currents were simultaneously sampled directly onto computer (at a frequency of 10 kHz) through a 2 kHz low-pass filter (8-pole Bessel, Frequency Devices, MA U.S.A.) and acquired with the program WinWCP (kindly supplied by Dr. J. Dempster, University of Strathclyde Electrophysiology Software: www.strath.ac.uk/Departments/PhysPharm/ses.htm). This program was also used to trigger the stimulator (used for evoking synaptic currents; see below) and to trigger the patch-clamp amplifier to deliver a 5mV test pulse after each pair of stimuli. Each recording sweep, recorded on-line by WinWCP, consisted of 20 ms of pre-stimulus baseline, the paired stimuli (plus any evoked currents) and a test-pulse response. A WCP data file for each cell was created containing all the recorded stimulation sweeps and responses, ready for further analysis.

Recordings of spontaneous miniature EPSCs were re-sampled from DAT tape at the end of each experiment. Sampling was performed in the same manner as described above,

however, the data for each cell was acquired as a continuous voltage-clamp recording of membrane current and was acquired using the program WinEDR (also supplied by Dr. J. Dempster, University of Strathclyde Electrophysiology Software). An inbuilt application of the program was then run that scanned the continuous trace and identified all fluctuations of the baseline current that passed the detection criteria for potential spontaneous synaptic events. The detection criteria were set to identify events that crossed a -3pA threshold for at least 0.7 ms. 102 ms stretches of recording, each containing a detected event, were then exported into WinWCP for further analysis.

2.6.6 *Paired-Pulse Stimulation of Unitary Glutamatergic EPSCs*

2.6.6.1 Extracellular Stimulation of Schaffer Collateral Axons

All experiments were performed in the presence of 6 μM SR95531, a $GABA_A$ receptor competitive antagonist.

Extracellular stimulating electrodes were made in an identical fashion to the recording electrodes, but were filled with a 1 M NaCl solution. The electrode holder contained a silver wire and was mounted upon a micromanipulator (Narishige, Japan). The ground for the stimulating electrode was a second piece of silver wire, coiled at its tip and submerged into the recording chamber solution.

The hippocampal slice was positioned within the recording chamber so that the CA1 region of the stratum pyramidale lay parallel with the horizontal axis of the microscope field of view – visualised on the video monitor (using a 40 x water immersion objective). The stage of the microscope was then manoeuvred in order to position the soma of the cell of interest into a corner of the monitor so that its apical dendrites and the Schaffer collateral axons of the stratum radiatum would occupy the majority of the screen. Before patching a cell, the stimulating electrode was positioned, touching the surface of the slice in the opposite corner of the monitor. The aim was to place the stimulating electrode within the stratum radiatum, whilst keeping it as far away as possible from the dendrites of the patched neurone; thereby, minimising the chance of stimulating the CA1 neurone directly or physically disturbing the whole-cell patch

whilst manoeuvring the stimulating electrode.

Once a cell was voltage-clamped, the position of the stimulating electrode was adjusted until a synaptic response could be elicited in the patched cell. Currents were evoked by applying pairs (inter-stimulus interval (ISI) 70 ms) of bipolar rectangular voltage pulses (150 μs duration, at a frequency of 0.2 Hz; GRASS SD9J square pulse stimulator, Astro-Med, Inc., RI, U.S.A). An inter-pulse interval of 70 ms was used for the determination of minimal stimulation (see below). Thereafter, the responses from at least 60 stimulation sweeps were collected using the 70 ms ISI..

2.6.6.2 Determination of Minimal Stimulation

Once a synaptic input was found, the position of the tip of the stimulating electrode was marked on the monitor and its location monitored throughout the experiment. In general, the stimulating electrode (as well as the recording electrode) was very stable and no visible drift occurred; however, if a slight movement did occur, its position was re-adjusted to ensure that the electrode continued to evoke synaptic currents arising from the input originally identified. The stimulation voltage of the paired pulses was returned to a sub-threshold intensity (i.e. the probability of evoking a postsynaptic current being zero) and then gradually increased until synaptic currents could be first observed. After 20 paired stimuli had been delivered, the voltage intensity was increased slightly and a further 20 stimuli delivered. Each set of 20 responses (including the stimuli where no synaptic response was detected – i.e. synaptic 'failures') were simultaneously averaged on an oscilloscope and the amplitudes of the paired EPSCs measured. The voltage was increased in this fashion until the average amplitude of the first EPSC had increased so that it appeared to be evoking a second input – i.e. the averaged peak amplitude had approximately doubled. In some recordings, it was not possible to recruit a further input without moving the electrode to a new location.

From these initial recording sweeps, an input/output relationship between stimulation intensity and the averaged current amplitudes was immediately generated and from this the stimulation intensity selected that was believed to reflect the stimulation of a single, or at most a few, presynaptic inputs (an example input/output relationship is shown in

figure 3.6).

2.6.6.3 Analysis of Evoked EPSCs

WinWCP was used for the analysis of minimally evoked EPSCs. To begin with, the baseline current level of each recording sweep was set through the mean of a 10 ms stretch of trace occurring before the first stimulation artefact. All current amplitudes were measured with respects to this baseline. Every individual recording sweep was checked by eye and sweeps remove that contained noisy current fluctuations that would falsely influence the mean response.

The peak amplitude of every response was plotted against time. This was done in order to confirm that the evoked currents were stable over the course of each recording, with no change in the range of their peak amplitudes or the pre-synaptic release probabilities. This served as an important check that minimal stimulation remained unaffected by either changes in the fidelity of the stimulating electrode, or patho-physiological changes occurring in the patched neurone. The peak amplitude of each test-pulse was also plotted as a function of time to ensure that the recording conditions did not undergo any significant change. Having ascertained a recording as stable, the mean peak amplitude (including failures) of the 1st and 2nd EPSC (EPSC1 and EPSC2 respectively) was measured and the paired-pulse ratio (PPR) calculated – defined as the mean peak amplitude of EPSC2 / EPSC1. Cells were also included in this analysis where the stimulation of more than one input was suspected.

The decay time constant (τ) of the averaged 1st EPSC was measured, using WinWCP, by fitting a single exponential to the decay phase of the averaged current, according to the equation:

$$I(t) = A.e^{(-t/\tau)} + C$$

Where, A is the fitted peak amplitude of the synaptic current, τ = time taken to decay to 36.8% from the peak amplitude, and C = the steady-state current when the event has fully decayed. The parameter C was fixed to zero, stipulating that the synaptic event

must fully decay back to the baseline zero current level.

For each minimal input, the probability that the first of each paired stimuli would fail to result in a synaptic current (probability of failures) was determined – detected by the absence of an evoked EPSC. This was achieved by visually inspecting each recording sweep and calculating the proportion of failures that occurred. For the average amplitude of EPSC1 and probability of failures data, cells were excluded if there was evidence that more than one input may have been stimulated; for example, quantal variation in individual EPSC1 amplitudes or differences in the latency or time course of evoked currents.

2.6.7 Spontaneous Miniature EPSC Recordings

All experiments were performed in the presence of 6 μM SR95531, a $GABA_A$ receptor competitive antagonist, and 1 μM TTX, a blocker of voltage-gated Na^+ channels which thus prevents action potential driven release of neurotransmitter. In some experiments, in order to confirm that the recorded mEPSCs were due to the activation of AMPA receptors, 10 μM of NBQX (an AMPA receptor competitive antagonist) was added during the latter half of the recording.

2.6.7.1 Analysis Miniature EPSCs

Analysis was limited to the first 360 seconds of each whole cell recording. As described above (section 2.6.5), the detection criteria used by WinEDR were set to identify events that crossed a -3pA threshold for a duration of 0.7 ms (figure 2.3 A). All detected events were carefully inspected by eye and events that possessed all of the synaptic-current selection criteria, (as described in figure 2.3 B) were selected as mEPSCs. The amplitudes of mEPSCs from this preparation were very small (ranging between 4 and 7pA), thus, in order to avoid the false selection of 'mini-like' events that were caused by random fluctuations in the baseline noise, the characteristics of detected 'mini-like' events recorded in the presence of 10 μM NBQX (an AMPA receptor antagonist) were studied. The selection criteria were therefore initially based upon the known properties of synaptic currents (points 1 and 2, figure 2.3 B) and then extended (points 3 and 4, figure 2.3 B) based upon the findings about random baseline current fluctuations. This

aided the distinction between glutamate receptor mediated synaptic events and synaptic current-like noise.

The peak amplitude of the each selected mEPSC was measured using WinWCP and the median amplitude and frequency of the mEPSCs that invaded each cell determined. For each recording, all selected miniature currents were subsequently averaged and the mEPSC rise-time of the averaged event assessed (again using WinWCP software). EPSC rise-time was calculated between data points corresponding to 20 and 80% of the peak amplitude.

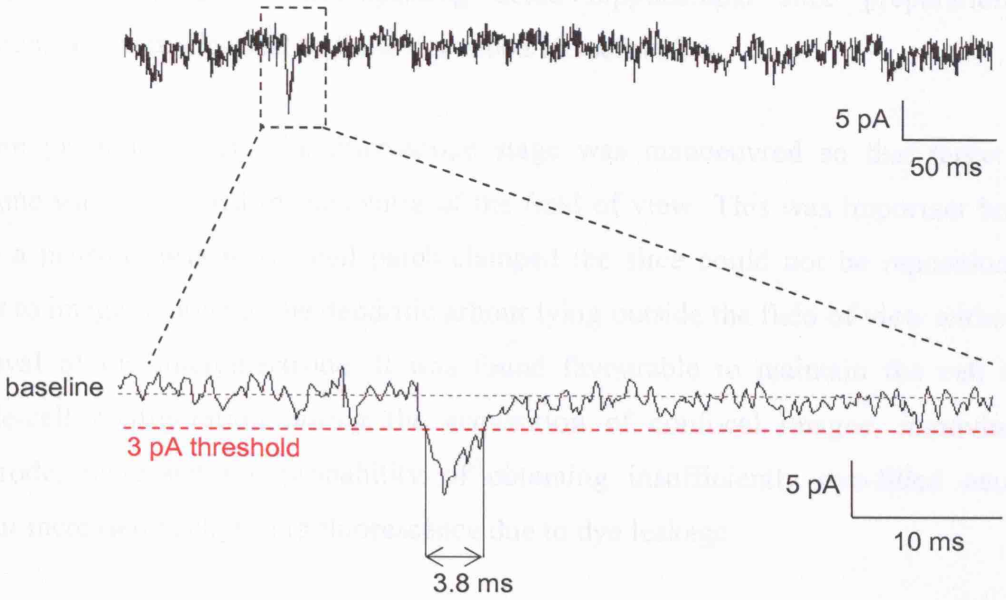
To assess the level of baseline noise of each recording, the mean standard deviation (s.d.) of the baseline current was assessed for each recording. Baseline current was analysed within a 5 ms time-window, starting 10ms before the onset of all detected events, and the mean s.d. value subsequently calculated for the entire record trace. This method does mean that the sections of trace lieing in-between each synaptic event were not assessed for noise; however, considering the detection parameters adopted (3 pA and 0.7 ms thresholds), sub-threshold noise would be unlikely to obstruct the detection of events.

Occasional stretches of more 'noisy' recording, caused, for example, by sources of external noise, were excluded from all analyses. Such noisy episodes were rare and generally very brief (usually in the order of ms).

Figure 2.3 Criteria for the detection and selection of miniature EPSCs

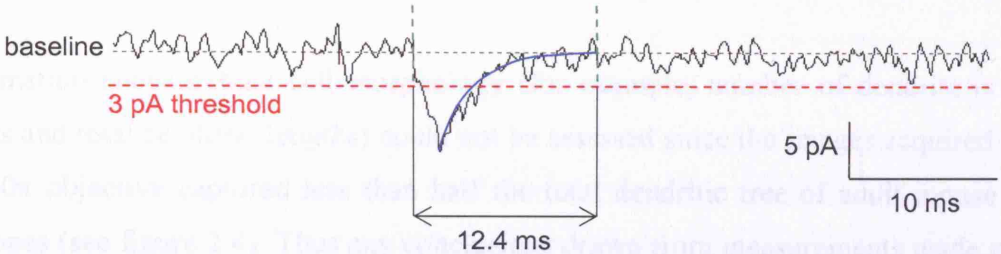
A) The *detection* of potential mEPSC events: Negative current deflections are detected that pass below a 3pA threshold for at least 0.7ms.

• This event remained below threshold for 3.8 ms



B) A detected event is only *selected* as a miniature EPSC if it adheres to the following visual selection criteria:
1 - Event decay time (shown in blue) is slower than the rise time
2 - Event must return to a stable baseline.
3 - Event peak amplitude is greater or equal to 4pA
4 - Event remains below baseline for at least 10ms.

• This event has an amplitude of 8.5pA and stayed below baseline for 12.4 ms.



2.7 Laser Scanning Confocal Microscopy

For dendritic spine visualisation, CA1 pyramidal cells of acute hippocampal slices were whole-cell patch-clamped with microelectrodes filled with intracellular solution (as used for the electrophysiological recordings; table 2.4) supplemented with the fluorescent dye, Alexa Fluor-594 (absorption and emission maxima: 588 and 613 nm respectively). Dye was added to the intracellular solution at a concentration of 0.2 mg/ml. All procedures encompassing acute hippocampal slice preparation and maintenance were identical to those described in section 2.6.

Before patching a cell, the microscope stage was manoeuvred so that target CA1 neurone was positioned in the centre of the field of view. This was important because once a neurone was whole-cell patch-clamped the slice could not be repositioned in order to image regions of the dendritic arbour lying outside the field of view without the removal of the microelectrode. It was found favourable to maintain the cell in the whole-cell configuration during the acquisition of confocal images; removing the electrode, increased the probability of obtaining insufficiently dye-filled neurones and/or increased background fluorescence due to dye leakage.

Images were obtained using an Olympus Fluoroview confocal microscope (generously supplied by Olympus, London, U.K.), mounted on an upright Olympus BX50WI using a 60x water-immersion objective (Olympus; numerical aperture, 0.9). Alexa Fluor-594 fluorescence was excited using an argon-krypton laser (excitation range, 457-576 nm), and collected using a 570 nm high-pass barrier filter. Confocal images were acquired as a series of optical z-sections. First, a crude image (step size 1.0 – 1.5 μm) was taken of the entire field of view (230 by 230 μm) to show the general morphology of the visible portion of the dendritic arbour. Afterwards, high resolution images (step-size 0.2 μm) of individual dendritic sections were taken under high-zoom (2.5 – 3.0) and all images were digitally stored for subsequent spine density analysis.

Information about overall cell morphology (for example, number of dendrite branch-points and total dendritic lengths) could not be assessed since the images acquired using the 60x objective captured less than half the total dendritic tree of adult mouse CA1 neurones (see figure 2.4). Thus any conclusions drawn from measurements made on

the visualized portion alone could be misleading. Acquiring adequate images of entire dendritic trees for morphology assessment requires removal of the electrode and repositioning the slice before further imaging. As the main objective of the study was to assess spine density in the dendritic regions that lay within the stratum radiatum and the optimal conditions for acquiring high-resolution images required leaving the electrode in place, images of entire dendritic trees were not made.

In a previous preliminary study, images of entire CA1 neurones from C57B6/J inbred wild-type and $CaMKII^{T286A}$ mutant mice (figure 2.4) were obtained using a Leica TCS SP2 laser-scanning spectral confocal microscope (courtesy of Dr. David Becker and the U.C.L. Department of Anatomy) and a 10x oil-immersion objective (Leica; numerical aperture, 0.4). In these cases, after a neurone was filled with dye, the electrode was removed and the slice placed into 4% paraformaldehyde diluted in 0.4 M phosphate buffer (PB: 4 parts 0.4 M Na_2HPO_4 plus 1 part 0.4 M NaH_2PO_4) and fixed overnight at 4 °C. The slice was then rinsed twice in fresh 0.4 M PB, and then mounted on a glass slide for microscopy.

Figure 2.4 CA1 Pyramidal Neurones Imaged with Confocal Micrscopy.

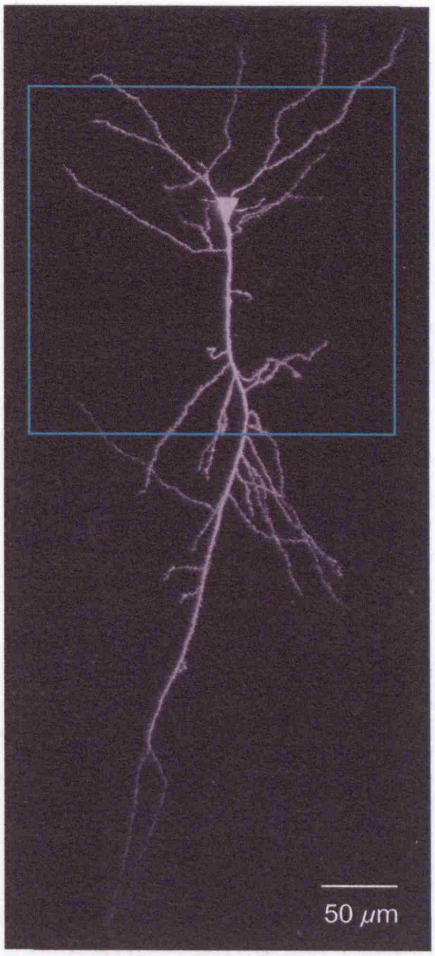
A (i) and B (i): Montage confocal images acquired using a 10x oil-immersion objective (numerical aperture, 0.4) showing the entire dendritic structures of fluorophore-filled CA1 pyramidal neurones (paraformaldehyde-fixed). Neurones are within adult wild-type and mutant mouse acute hippocampal slices (A and B respectively).

A (ii) and B (ii): These montage confocal images were acquired using a 60x water-immersion objective (numerical aperture, 0.9) and are of live (i.e. not fixed) fluorophore-filled CA1 pyramidal neurones from adult wild-type and mutant mouse acute hippocampal slices (A and B respectively).

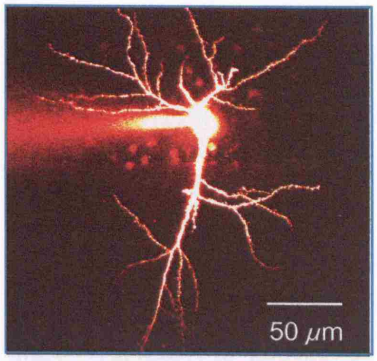
These images demonstrate the extent of the CA1 dendritic arbours that can be visualised within the 60x objective field of view. Less than half the apical dendritic tree can be captured. All images shown are of cells obtained from mice raised in standard housing conditions.

A) Wild-Type

i)



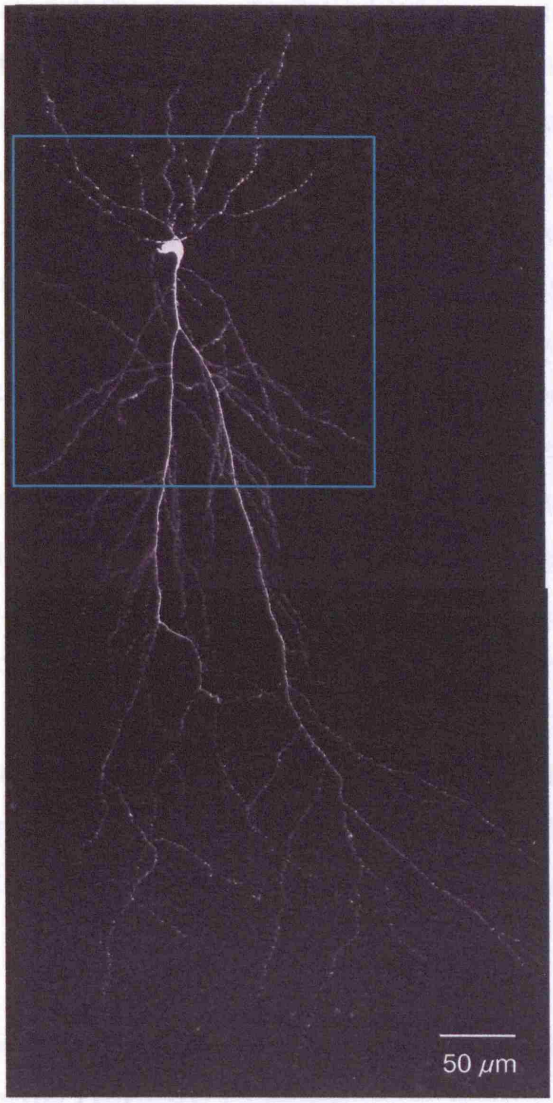
ii)

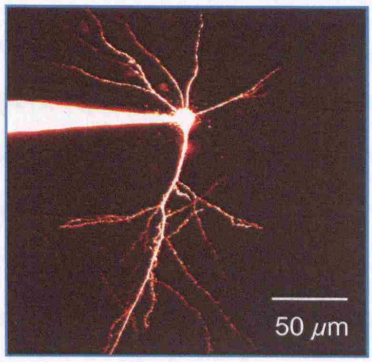


B) Mutant

i)

ii)





2.7.1 Quantitative Spine Density Analysis

All confocal experiments were conducted and the images analysed, blind to the mouse genotype and housing condition. Spine counts were made using Image J software (National Institute of Health, U.S.A.). Counting was performed manually by studying the single xy composite images through the consecutive planes of the z-series. The spine densities (spines per μm of dendrite) were calculated in images obtained using a 60x objective combined with a high-zoom (2.5 – 3.0). Spine density equals the total number of spines counted along a section of dendrite divided by the length of the section. Section length was calculated, using Pythagoras theorem, from the dendritic section length (measured in x-y plane) and the corresponding depth through which the section spanned in the z plane. Since 3D reconstructed images of the cells were not made, it was difficult to accurately count spines that lay either directly above or below the dendrite; such spines were therefore excluded from the counts even when some were clearly visible as being in such positions. While this will have resulted in a small reduction in calculated spine density, the extent of reduction is likely to be proportional to the total spine density and will have affected all cells equally. The apical spine counts were calculated from dendritic images of the secondary branches (those that arose directly from the primary apical dendrite) and tertiary branches (arising from secondary dendrites). The radial distance of each imaged region from the soma was measured and only regions that lay within 2 standard deviations of the mean distance were analysed. The final radial distance range was 28-138 μm. (All apical images from one cell, plus one dendritic section from another, were removed from further analysis based upon this criterion).

2.8 Statistical Examination of Electrophysiological Data and Spine Density Counts

The data for each experimental animal group are presented as means ± standard error of the mean (s.e.m.) Cells with values more than two standard deviations away from the mean (for that experimental group) were omitted from the group averages and all statistical analyses. This never resulted in more than one cell being omitted per group. The number of data points per group (n number) refers to the number of cells analysed. No more than one cell was recorded from or imaged for each individual hippocampal

slice; group data were collected from at least n=3 animals. The statistical tests used assume that the data are sampled from populations that follow Normal distributions. If a data group did not follow a normal distribution (tested for using the Kolmogorov-Smirnov normality test), then the data groups under statistical scrutiny were log-transformed before parametric statistical tests were then carried out. This was only necessary do to for analysis of EPSC1 averaged amplitudes as the wild-type data from standard environments did not follow a normal distribution ($P = 0.004$). Two-way t-tests, ANOVA and the Kolmogorov-Smirnov normality test were carried out using the statistical computer package PRISM, version 4 (GraphPad software Inc.).

For the initial experiments performed (i.e. figure 3.7 – recordings of minimally evoked synaptic currents in wild-type and mutant animals raised in the standard housing environment only), statistical scrutiny was carried out using two-tailed t.tests. To test whether the variances of the data, from the two populations, were different, F tests were also performed. The F test generates a P value that answers the question of whether the difference between the variances of the two populations may have occurred by chance. For both F tests and t.tests, values of $P < 0.05$ were considered significant.

Differences between the four experimental animal groups were statistically examined using analysis of variance (ANOVA). Again, values of $P < 0.05$ were considered significant. If significant differences were detected, Bonferroni post-tests were also carried out, to further characterise the group differences. The Bonferroni method applies a correction accounting for the multiple comparisons; thus, ensuring that the 5% probability applies across all the comparisons contemporarily and not to each individual comparison. Non significant P values for the Bonferroni post-tests are given at values greater than 0.05. Whereas, P values implicating significant or very significant effects of environmental enrichment are given as $P < 0.05$ or $P < 0.01$ respectively (as provided by PRISM).

To investigate whether the distribution of data were affected by enrichment, cumulative faction graphs were plotted and the probability that the two distributions were drawn from the same population statistically tested using the two-sample Kolmogorov-Smirnov test. The computer program used to perform this test was kindly supplied by Dr. Gerald Finnerty of the Institute of Psychiatry, Department of Neurology, Kings

College London. P values less than 0.05 were again considered significant and the null hypothesis rejected.

For correlation analyses, Pearson correlation calculations were performed. For each calculation, the correlation coefficient of determination, r^2, and the two-tailed P value is presented. The fraction of variance in one variable that can be explained by variance in the other is indicated by the value of r^2. Whereas, the P value determines the probability that the correlation occurred through random sampling (i.e. a P value ≤ 0.05 rejects the null hypothesis which states that there is no correlation in the overall population and any correlation within the data occurred by chance).

Correlations were performed upon the data points both within each the four animals groups separately and with all groups combined together.

2.9 Drugs and Solutions

The composition of the modified Krebs solution used during brain slicing and the standard Krebs solution used throughout experiments thereafter are given in table 2.3. All chemicals were obtained from VWR International (U.K.). The Krebs solutions were made up either on the day of the experiments, or, in the afternoon of the previous day.

The composition of the intracellular solution is given in table 2.4. Aliquots of the intracellular solution (0.5 ml) were made up and stored at -20 °C. When required, an aliquot was thawed and then kept on ice throughout the course of an experiment.

SR95531 and 7-chlorokynurenic acid (7ClK) were dissolved in distilled water whereas QX-314,2.3-dioxo-6-nitro-1,2,3,4-tetrahydroquinoxaline-7-suphonamide (NBQX) was dissolved in dimethylsulphoxide (DMSO). All were obtained from Tocris (Bristol, U.K.). Each drug was prepared at 1000 times its final concentration and all concentrated aliquots were stored at -20 °C. Aliquots were thawed on the day of the experiment and, when required, diluted into the slicing or standard Krebs solutions to achieve the required concentration.

The fluorescent dye, Alexa Fluor-594, was obtained from Molecular Probes (OR,

U.S.A), and dissolved in intracellular solution at a concentration of 1.0 mg/ml. This stock solution was filtered and 50 µl aliquots of the filtrate stored at -20 °C. On the day of an imaging experiment, an aliquot was thawed on ice and 200 µl of freshly thawed intracellular solution added giving a final dye concentration of 0.2 mg/ml.

Table 2.3 Composition of Slicing and Standard Krebs Solutions.

Chemicals	Slicing Krebs (mM)	Standard Krebs (mM)
NaCl	85	125
KCl	2.5	2.5
$NaH_2PO_4.H_20$	1.25	1.25
$NaHCO_3$	25	26
$CaCl_2$	0.65	2
MgCl	4	1
Glucose	25	25
Sucrose	75	0
NBQX	5μM	-
7ClK	5μM	-

Table 2.4 Composition of Intracellular Pipette Solution.

Chemicals	Intracellular Solution (mM)
CsCl	140
$CaCl_2$	2
HEPES	5
EGTA	10
Mg-ATP	2
pH adjusted with CsOH to 7.4	

CHAPTER 3
Results

The first section of the results describes the distribution of αCaMKII mRNA in the mouse brain during early postnatal development and at adulthood and investigates whether the mRNA expression pattern is altered in the αCaMKIIT286A mutant mouse. The following sections address whether a life in the absence of all neuronal processes that depend upon the autophosphorylation of αCaMKII at residue Thr286, like NMDA receptor-dependent LTP, affects either the basal properties of evoked synaptic transmission at unitary Schaffer collateral to CA1 synapses or properties of the total excitatory input onto individual CA1 neurones. Experiments were performed in mice raised in standard laboratory housing environments as well as in mice that received post-weaning environmental enrichment – a procedure that was used to promote hippocampal-dependent plasticity.

3.1 The Expression of αCaMKII in Wild-type and Mutant Mouse Brain through Post-Natal Development

3.1.1 Introduction

There were two aims for this section of the thesis:

The first aim was to determine when during postnatal developmental the α-isoform of CaMKII was first expressed in wild-type mouse brain. The second, and most important aim for the subsequent investigations, was to assess whether the expression patterns of αCaMKII through development were affected by the CaMKIIT^{286A} altered gene.

The plasticity of connections is an important process recruited during brain development (e.g. Goodman and Shatz, 1993). Thus, an understanding of the developmental profile of αCaMKII expression may help understand when and where in the brain αCaMKII-dependent processes could start to come into play and influence

brain neurochemistry and function, including synaptic plasticity. Identifying the onset of expression and the age at which adult-like αCaMKII intensities are reached are therefore important in order to understand how the lack of CaMKII-dependent functions might be able to affect brain maturation in the mutant mice. The age at which adult-like intensities of αCaMKII protein are attained in the brain has been previously studied (Kelly and Vernon, 1985;Kelly et al., 1987;Burgin et al., 1990) and was therefore not an aim of the present study. However, it has not yet been clearly demonstrated when and to what extent αCaMKII is first expressed during brain development. One study that looked at the expression of all four CaMKII isoforms at various pre- and post-natal stages could only detect αCaMKII in postnatal brain (Bayer et al., 1999). This was in marked contrast to the β-, δ- and γ-isoforms which all showed embryonic onsets of expression. Clear labelling of αCaMKII mRNA has, however, been demonstrated in P4 animals with the strongest signal being present in the hippocampus (Burgin et al., 1990). For this thesis, I specifically looked, therefore, at each postnatal day previous in order to identify the first day of expression.

With respects to the second aim, it has already been shown that in whole adult mouse brain the protein levels of α and of β CaMKII are comparable between homozygote wild-types and mutants (Giese et al 1998; mice aged 5 - 10 months). It has also been demonstrated that there are no differences between the genotypes at adulthood in the hippocampal levels of αCaMKII protein expression (Hardingham et al., 2003). However, there has been no work to confirm that the there are also no differences in level or pattern of expression during earlier stages of development. Discrepancies in either the onset of expression or the distribution of αCaMKII in young animal could reflect differences in brain physiology and/or the initiation of compensatory mechanisms that would have important implications upon the interpretation of data gathered at adulthood (6-8 weeks in these studies). Thus, although changes in these parameters are not expected, it serves as an important control for the later studies into hippocampal physiology, strengthening any evidence that implicates new information about the function of αCaMKII Thr286 autophosphorylation.

To address these aims, we used *in situ* hybridization and immunocytochemical techniques to respectively detect αCaMKII mRNA and protein in wild-type and mutant

mouse brain. The *in situ* hybridization study was carried out in collaboration with Dr. Florentina Soto of the Max-Planck-Institut für Experimentelle Medizin, Göttingen, Germany. The wild-type and mutant mouse tissue preparation was prepared at U.C.L. and then transported to Germany for subsequent hybridization and autoradiography. The immunocytochemistry was carried out within the Department of Anatomy at U.C.L. with the kind help and advice from Professor Maria Fitzgerald and postgraduate student Damian Patterson.

3.1.2 The Detection of αCaMKII mRNA

Two distinct antisense oligonucleotide probes (probes A and B) against the αCaMKII mRNA molecule were designed by Dr. Soto in Germany. In addition, a sense probe, the complement of probe A, was also used as a tool to detect non-specific binding. Control brain sections were hybridised in the presence of 500-fold excess unlabelled ('cold') probe.

No positive alignment matches, other than the murine αCaMKII mRNA, were found for any of the probes using the BLAST® search procedure. All the major non-redundant sequence data bases (GenBank, EMBL, DDBJ and PDB) were searched.

3.1.3 The In Situ Hybridization Signal is Specific

Figure 3.1 demonstrates the three control measures considered in order to conclude that the hybridisation signal was specific and indicative of the distribution of αCaMKII mRNA in the mouse brain. The adult brain sections show the most dense hybridisation signal and are presented here to demonstrate the relative specificity of the probes. All three controls were used at all postnatal ages and for both wild-type and mutant brain sections.

The first test for specificity was the use of the anti-sense control sections in which the excess of cold probe (500-fold) was able to fully compete with the labelled probe for the binding sites. This form of specificity control is widely used for *in situ* hybridization studies (e.g. Stocker and Pedarzani, 2000). This excess of cold-probe would not be expected to fully displace the radiolabelled probes if the anti-sense probes were also

labelling a large proportion of non-specific binding sites in the brain tissue. The second test for non-specific binding was the use of the sense radiolabelled probe. As shown in figure 3.1, both the anti-sense and sense control sections showed uniformly low non-specific binding.

Finally, the use of two distinct antisense probes that bind to the αCaMKII mRNA molecule at non-overlapping locations (probe A, nucleotides 80-129, and probe B, nucleotides 1074-1120) also acts as a control for specificity. Figure 3.1, shows that identical hybridization patterns were obtained with the two probes supporting the conclusion that the hybridisation signal is specific in each case and ruling out the possibility of spurious cross-reactivity from either oligonucleotide. It can also be seen that the staining of probe B is notably denser than that of probe A; this was evident at all ages studied (data not shown).

All three controls were used at all the postnatal ages studied and for both wild-type and mutant brain sections. Additionally, all hybridisations were generated from a single experiment; all brain sections underwent simultaneous and identical treatments and a single batch of each probe was used throughout. Furthermore, the corresponding experimental and control autoradiographs for a particular probe at each age were generated by exposure to the same X-ray film. However, it was not possible to expose all slides from all ages to the same film, thus, qualitative but not quantitative comparisons can be made across the ages with respects to staining densities.

The brain sections shown are representative of each experimental condition at each age and for each genotype. Experimental and corresponding control hybridizations of the probes were performed in brain sections taken from the same animal; 6-8 brain slices were hybridized for each of the probes per genotype, at each age and for both experimental and control conditions. Different mice were used for the two antisense probes. 3-4 brain slices were hybridized with the sense probe for each of the genotypes at each age studied.

Three weeks exposure to the X-ray film produced saturating autoradiography levels of staining as revealed by the optical density scale bar. This was the case for both probes. However, the extent of saturation in the brain was less for probe A (see figure 3.1).

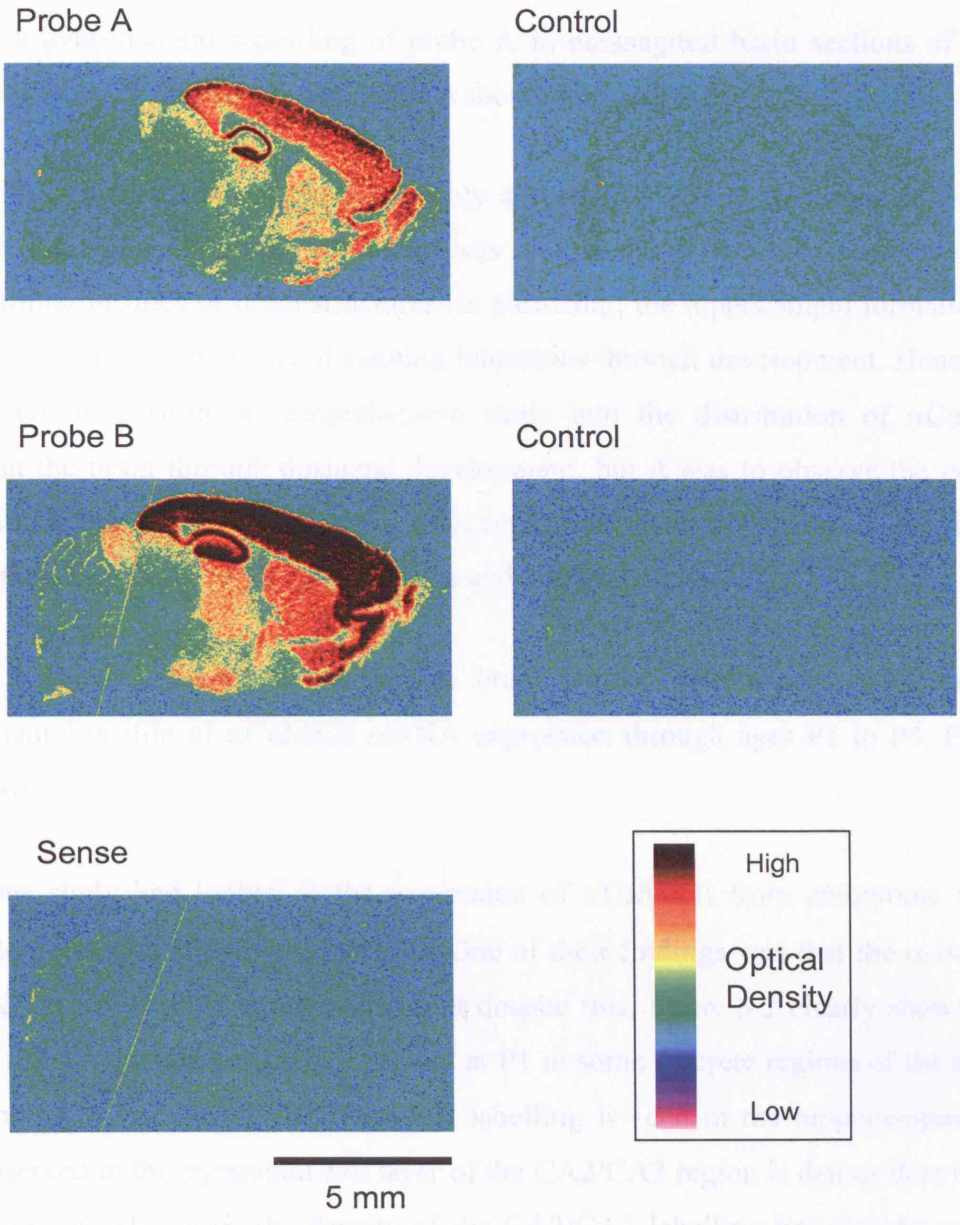
Whilst this also prevents quantitative comparisons of saturated sections to be made (for example, in the hippocampus), the pattern of expression can still be qualitatively compared. The brain sections for probe A are therefore used throughout the rest of this results section.

Figure 3.1 CaMKII *In Situ* Hybridization Controls.

These adult brain sections show the hybridisation signals obtained with two different anti-sense oligonucleotides, probes A and B. The pseudo-colour scale shows optical density which correlates with the level of probe labelling.

The anti-sense control sections were hybridised in the presence of a 500-fold excess of cold probe and show very low levels of non-specific probe binding. The identical hybridization patterns obtained with the two different probes support the conclusion that the signal is specific for αCaMKII. A brain section hybridised with the sense probe (the complement of probe A) is also presented and again demonstrates minimal levels of non-specific binding.

A sketched profile of an adult mouse parasagittal brain slice is presented with the brain structures that show the highest levels of αCaMKII mRNA hybridization signal indicated.


Probe A
Control
Probe B
Control
Sense
High
Optical Density
Low
5 mm
Key to brain regions
IC
H
FC
C
T
CP
O
CP Caudate Putamen
C Cerebellum
FC Frontal Cortex
H Hippocampus
IC Inferior Colliculus
O Olfactory Bulb
T Thalamus

3.1.4 *The Expression and Distribution of αCaMKII mRNA in Wild-Type Mouse Brain*

The *in situ* hybridization signalling of probe A in parasagittal brain sections of wild-type mouse from P1 through to adulthood is shown in figure 3.2.

As described in methods section 2.4.1, only a restricted region of mouse brain was sectioned. The aim of limiting the region was so that the selection of sections would contain similar profiles of brain structures (in particular, the hippocampal formation) in order to make fair comparisons of staining intensities through development. Hence, the aim was not to perform a comprehensive study into the distribution of αCaMKII throughout the brain through postnatal development, but it was to observe the general changes in expression that occur in the brain regions of interest – i.e. the major sites of synaptic plasticity, such as the hippocampus and cerebral cortex.

Figure 3.2 shows a series of comparable brain sections demonstrating the general developmental profile of αCaMKII mRNA expression through ages P1 to P4, P8 and adult (P36).

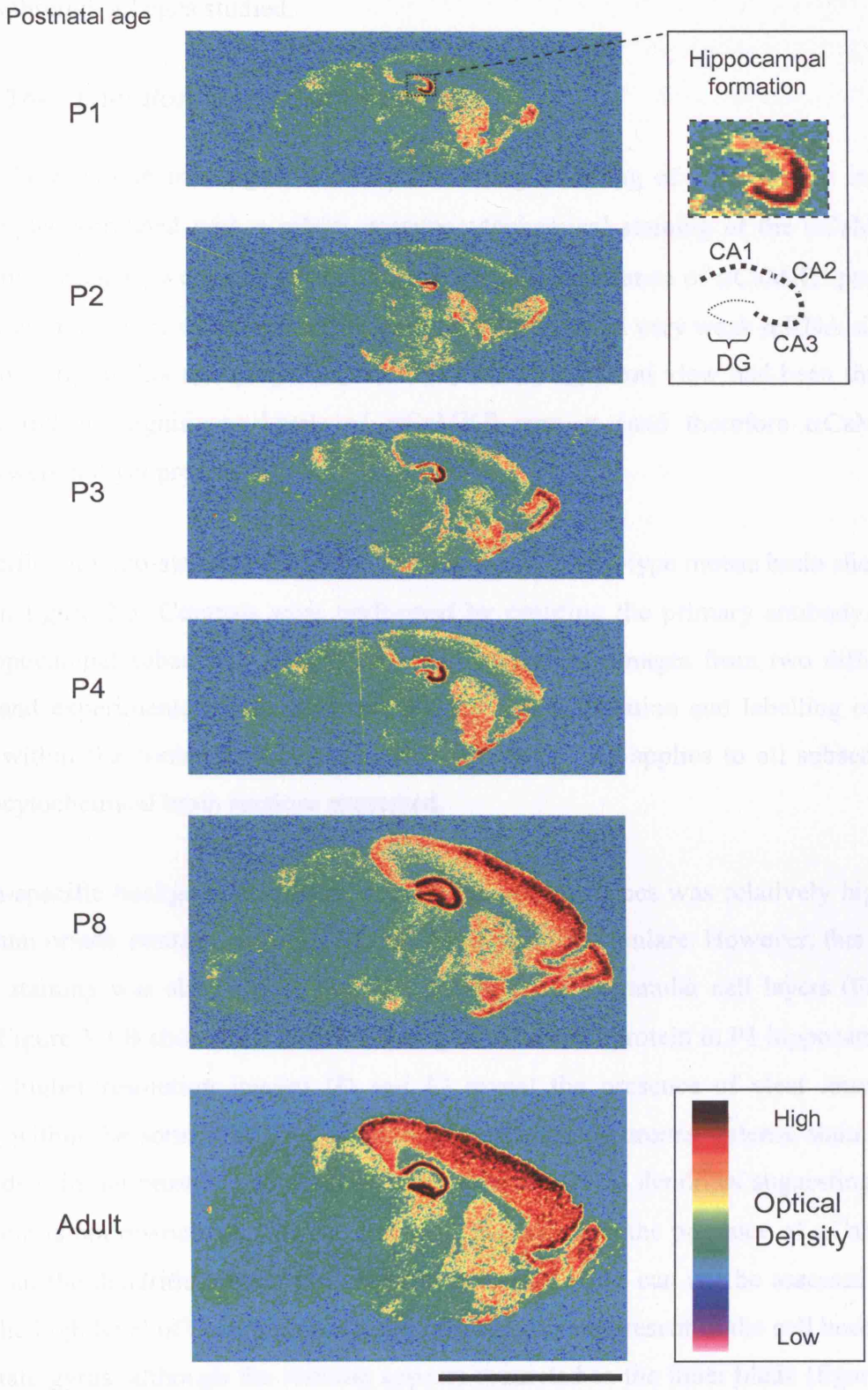
A previous study had looked at the expression of αCaMKII from embryonic stages through to adulthood (Bayer et al., 1999). One of their findings was that the α-isoform of CaMKII is not expressed prenatally. Yet despite this, figure 3.2 clearly shows high levels of mRNA expression already present at P1 in some discrete regions of the mouse brain. The most intense αCaMKII mRNA labelling is seen in the hippocampus. The signal observed in the pyramidal cell layer of the CA2/CA3 region is denser than that of the CA1 region. However, the density of the CA2/CA3 labelling has already reached maximal detection levels, thus, we are unable to see whether staining in this region increases further through development. In the dentate gyrus, only the granule cells of the inner blade are labelled. Other regions labelled at P1, include the caudate putamen, the olfactory bulb and the entorhinal cortex.

Signalling in the CA1 and dentate gyrus increases between P1 and P4, and adult like staining throughout the hippocampus and dentate gyrus is seen by P8. With respects to

Figure 3.2 The Distribution of αCaMKII mRNA in Wild-Type Mouse Brain through Postnatal Development.

Example wild-type mouse brain sections of increasing age are presented; they show the distribution of αCaMKII mRNA at postnatal day 1 (P1), P2, P3, P4, P8 and P38 (adult). An expanded image of the hippocampal formation from P1 mouse is shown. Below is a schematic drawing indicating the respective pyramidal and granule cell layers of the hippocampus and dentate gyrus (DG). The hippocampal sub-regions CA1, CA2 and CA3 are labelled.

The pseudo-colour scale indicates optical density correlating with mRNA labelling intensity is shown.


Postnatal age
P1
P2
P3
P4
P8
Adult
Hippocampal formation
CA1
CA2
CA3
DG
High
Optical Density
Low
5 mm

labelling in the cerebral cortex, low levels of αCaMKII mRNA are present in the most superficial layers during the first few days of postnatal life and the intensity of staining increases through all ages studied.

3.1.5 *The Detection of αCaMKII Protein*

The aim here was to investigate whether the strong labelling of mRNA seen in the hippocampus correlated with a robust immunocytochemical staining of the αCaMKII protein. In particular, we wanted to verify the potential abundance of αCaMKII protein in the P1 animals since a previous report could only detected a very weak mRNA signal for this isoform at this age (Bayer et al., 1999) and the general view had been that in newborn rodents significant levels of αCaMKII protein (and therefore αCaMKII activity) were not yet present.

The specific immuno-staining of αCaMKII protein in P1 wild-type mouse brain slices is shown in figure 3.3. Controls were performed by omitting the primary antibody. For each hippocampal subsection imaged at higher resolution, images from two different control and experimental brain slices are shown. The orientation and labelling of the regions within the control hippocampal slice (figure 3.3 A) applies to all subsequent immunocytochemical brain sections presented.

The non-specific background staining seen in the control slices was relatively high in the stratum oriens, stratum radiatum and the lacunosum moleculare. However, this non-specific staining was absent from both the pyramidal and granular cell layers (Figure 3.3 A). Figure 3.3 B shows the overall staining of αCaMKII protein in P1 hippocampus, and the higher resolution images (D and E) reveal the presence of clear immuno-staining within the somata of CA1 and CA2/3 pyramidal neurones. Intense staining is also evident in the proximal sections of some primary apical dendrites suggesting that the protein is not restricted to the cell bodies. Unfortunately, the presence of αCaMKII throughout the dendritic arbour of pyramidal neurones at P1 can not be assessed here due to the high level of background staining. Protein is also present in the cell bodies of the dentate gyrus, although the staining appears restricted to the inner blade (figure 3.3 F). All these observations correlate well with the distribution of labelled mRNA in the

P1 hippocampus (figure 3.2 and 3.3 C)

The somata of CA1 pyramidal and dentate granule cells appear to be labelled less intensely than that of the pyramidal cells from the CA2/CA3 sub-regions. Again, this correlates well with the pattern of mRNA staining seen with *in situ* hybridization (figure 3.3 C).

The overall pattern of αCaMKII protein staining at P4 (figure 3.4 A, B, D-F) appears to be generally similar to that of the P1 brain slices; this again correlates well with the distribution of the mRNA hybridization signal (figure 3.4 C). The overall intensity of specific and non-specific background staining is less in these slices, allowing some qualitative observations to be made about the level of staining within the stratum oriens, the stratum radiatum and the lacunosum moleculare. It can be seen that the low intensity of background staining between these regions is continuous in the control brain section (figure 3.4 A), however, in the experimental section the level of staining within the equivalent strata of regions CA2 and CA3 is more intense than that seen in the CA1 sub-region. This supports the supposition that CaMKII protein is also located within the dendrites of the neurones at P4 and is not restricted to the cell bodies.

Figure 3.3 Immunocytochemical Staining of αCaMKII Protein in P1 Mouse Hippocampus.

A) A P1 control hippocampal brain section showing the obtained levels of non-specific DAB staining. With the exception of the pyramidal and granule cell body layers, levels of non-specific staining were high. Hippocampal sub-regions (CA1, CA2 and CA3) and the dentate gyrus are labelled. The following sub-strata are also indicated: *stratum oriens* (**o**); *stratum pyramidale* (**p**); *stratum radiatum* (**r**); *stratum lacunosum moleculare* (**m**). The inner (**i**) and outer (**o**) blades of the dentate gyrus are also labelled.

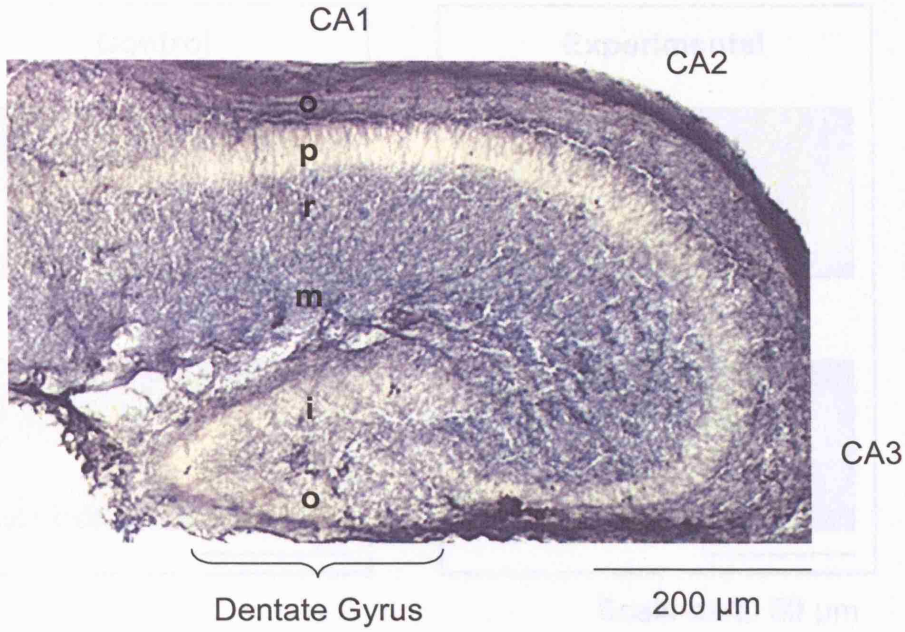
B) Specific αCaMKII protein staining in P1 hippocampus. Specific staining is seen in the stratum pyramidale of CA2 and CA3, and to a less extent within CA1 and granule cell layer of the inner blade of the dentate gyrus.

C) Example *in situ* hybridisation images of P1 hippocampus labelled for αCaMKII mRNA. The pattern of mRNA expression seen in these sections is similar to the pattern of protein staining as seen in B); this supports the conclusion that αCaMKII immunoreactivity and DAB staining is specific.

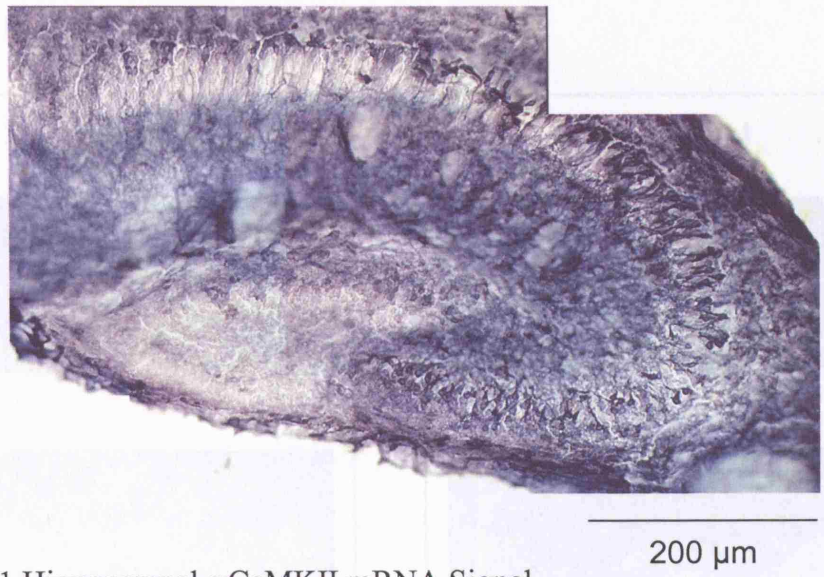
D – E) High resolution images of hippocampal neurones from 2 separate control and experimental P1 brain sections. Images show the respective levels of non-specific (controls) and specific (experimental) labelling of αCaMKII protein in the pyramidal cells of the CA1 (D) and CA2/3 (E) hippocampal sub-regions.

F) Images of P1 mouse dentate gyrus from 2 separate control and experimental brain sections. In the experimental sections, enlarged regions of the inner and outer blades demonstrate the higher levels of specific labelling found in the granule cells of the inner blade. This finding also corresponds with the mRNA staining patterns as seen in C).

A) P1 Control Section

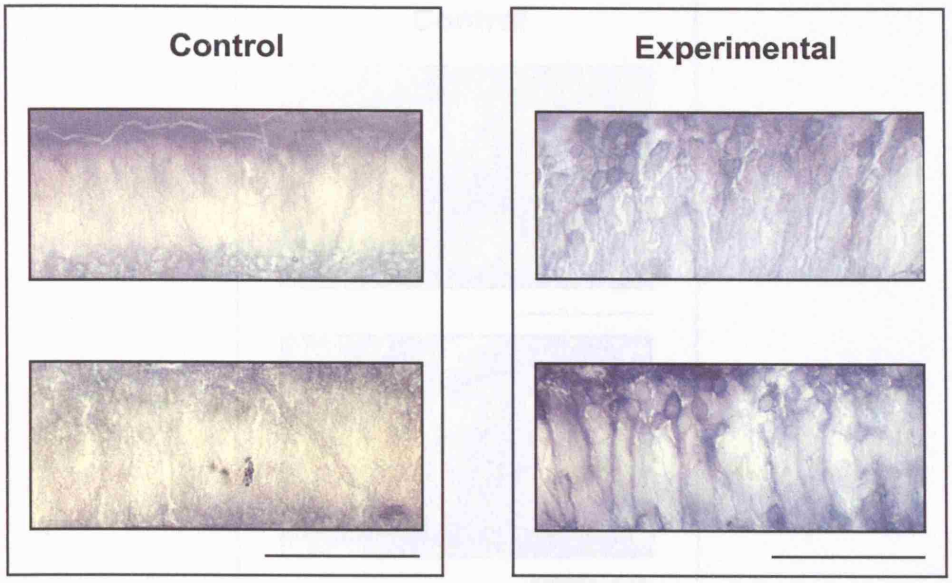


B) P1 αCaMKII Experimental Section



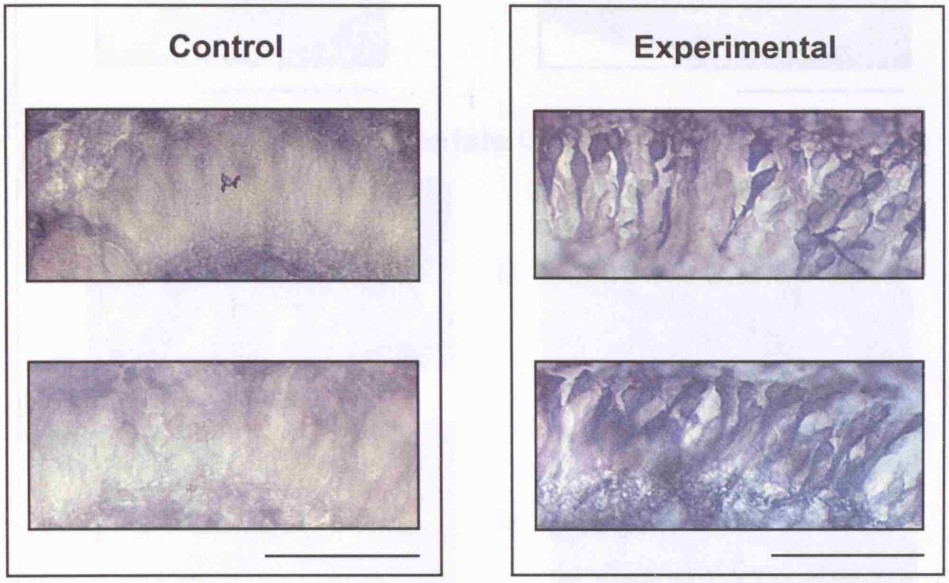
c) P1 Hippocampal αCaMKII mRNA Signal

D) P1 CA1 Pyramidal Neurones



Scale bars: 50 µm

E) P1 CA2/3 Pyramidal Neurones



Scale bars: 50 µm

F) P1 Dentate Gyrus

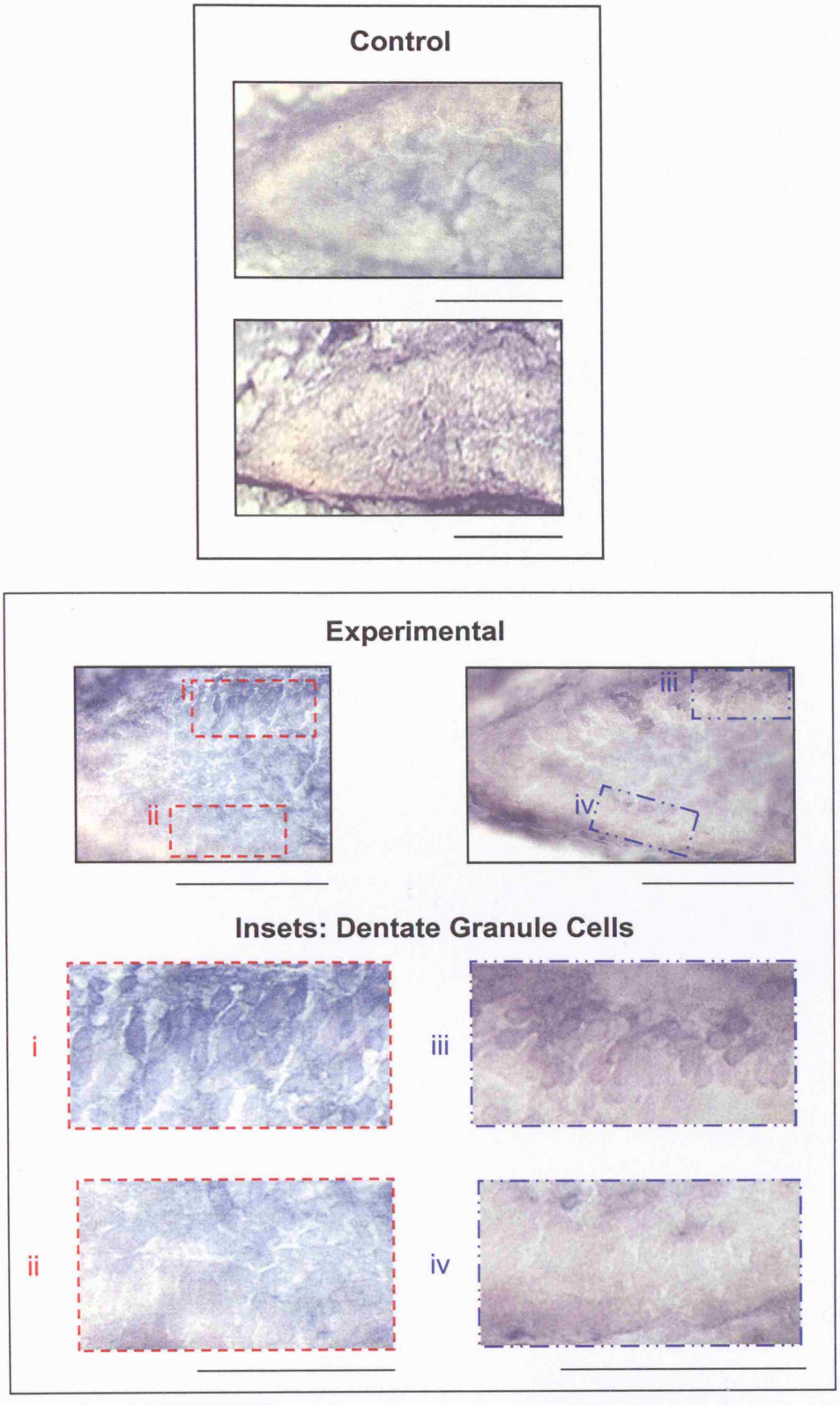


Figure 3.4 Immunocytochemical Staining of αCaMKII Protein in P4 Mouse Hippocampus.

A) A control P4 hippocampal section showing non-specific DAB staining. Non-specific staining in the P4 sections was relatively low.

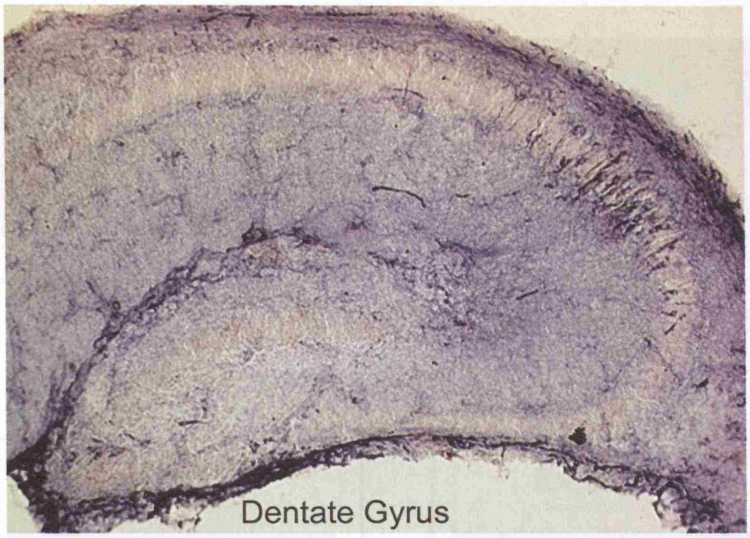
Note, in this specific section a discrete region of non-specific staining can be seen within the pyramidal cell layer of CA2/3 (possibly capillary vessels).

B) Specific αCaMKII protein staining in P4 hippocampus. Specific staining is seen in pyramidale stratae of CA2 and CA3 and to a less extent within CA1 and the granule cell layer of the inner blade of the dentate gyrus – a similar pattern as seen at P1.

C) These example *in situ* hybridisation images of αCaMKII mRNA in P4 hippocampus show a similar staining pattern to the protein signal as seen in B). This supports the conclusion that αCaMKII antibody immunoreactivity and DAB staining is specific.

D – F) High resolution images of hippocampal neurones from a control and an experimental P4 brain section. Images show respective levels of non-specific (control) and specific (experimental) labelling of αCaMKII protein in the pyramidal neurones of the CA1 (D) and CA2/3 (E) hippocampal sub-regions and in granule cells of the inner blade of the dentate gyrus (F and inset).

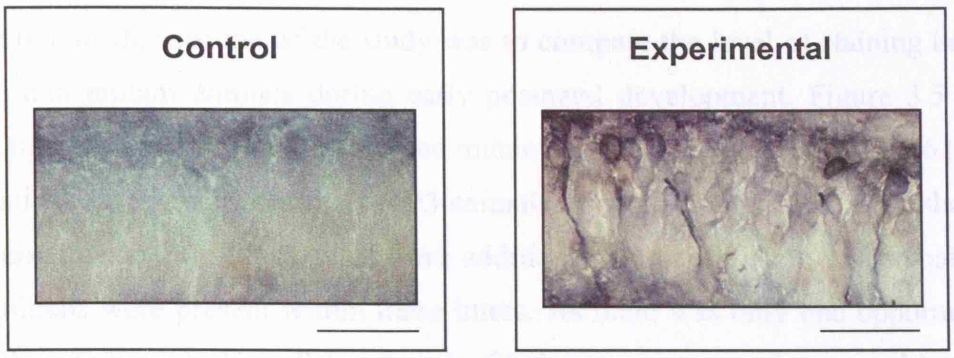
A) P4 Control Section



B) P4 αCaMKII Experimental Section

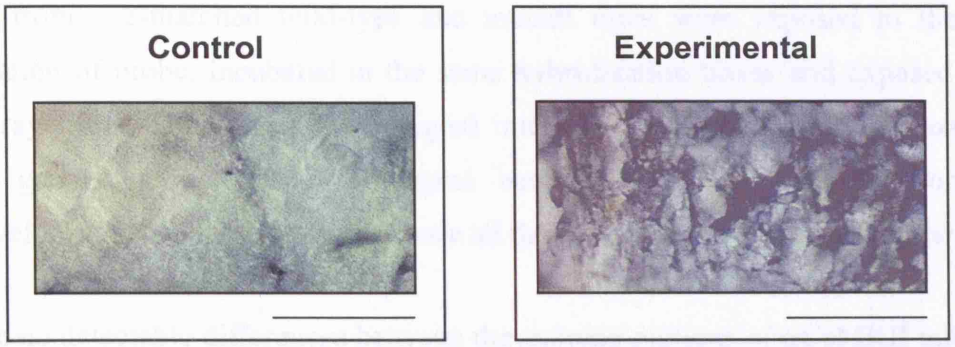
C) P4 Hippocampal αCaMKII mRNA Signal

D) P4 CA1 Pyramidal Neurones



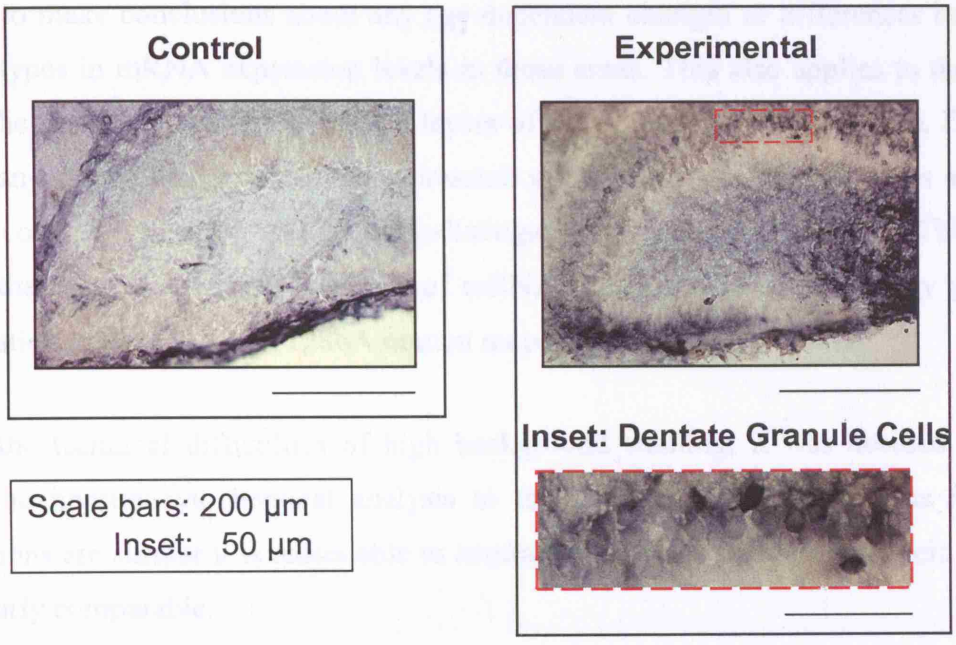
Scale bars: 50 μm

E) P4 CA2/3 Pyramidal Neurones



Scale bars: 50 μm

F) P4 Dentate Gyrus



Scale bars: 200 μm
Inset: 50 μm

3.1.6 *The T286A Point Mutation does not affect the Developmental Pattern of αCaMKII mRNA Expression*

The objective of this section of the study was to compare the level of staining between wild-type and mutant animals during early postnatal development. Figure 3.5 shows representative sections from wild-type and mutant animals aged P1, P4 and P36 (adult). Hybridisations were also performed in P3 animals of each genotype (data not shown as indistinguishable to P4); P8 animals were additionally processed but unfortunately no mutant animals were present within these litters. As there was only one opportunity to perform this study with the collaborators in Göttingen, Germany, I was unable to wait for further animals, so regrettably the comparison remains incomplete at this age.

Sections from age-matched wild-type and mutant mice were exposed to the same concentration of probe, incubated in the same hybridization boxes and exposed to the same X-ray films. Thus, the relative signal intensities at each age can be compared between genotypes. However, the signal between ages can only be compared qualitatively, as it was impossible to expose all the brain sections to a single X-ray film.

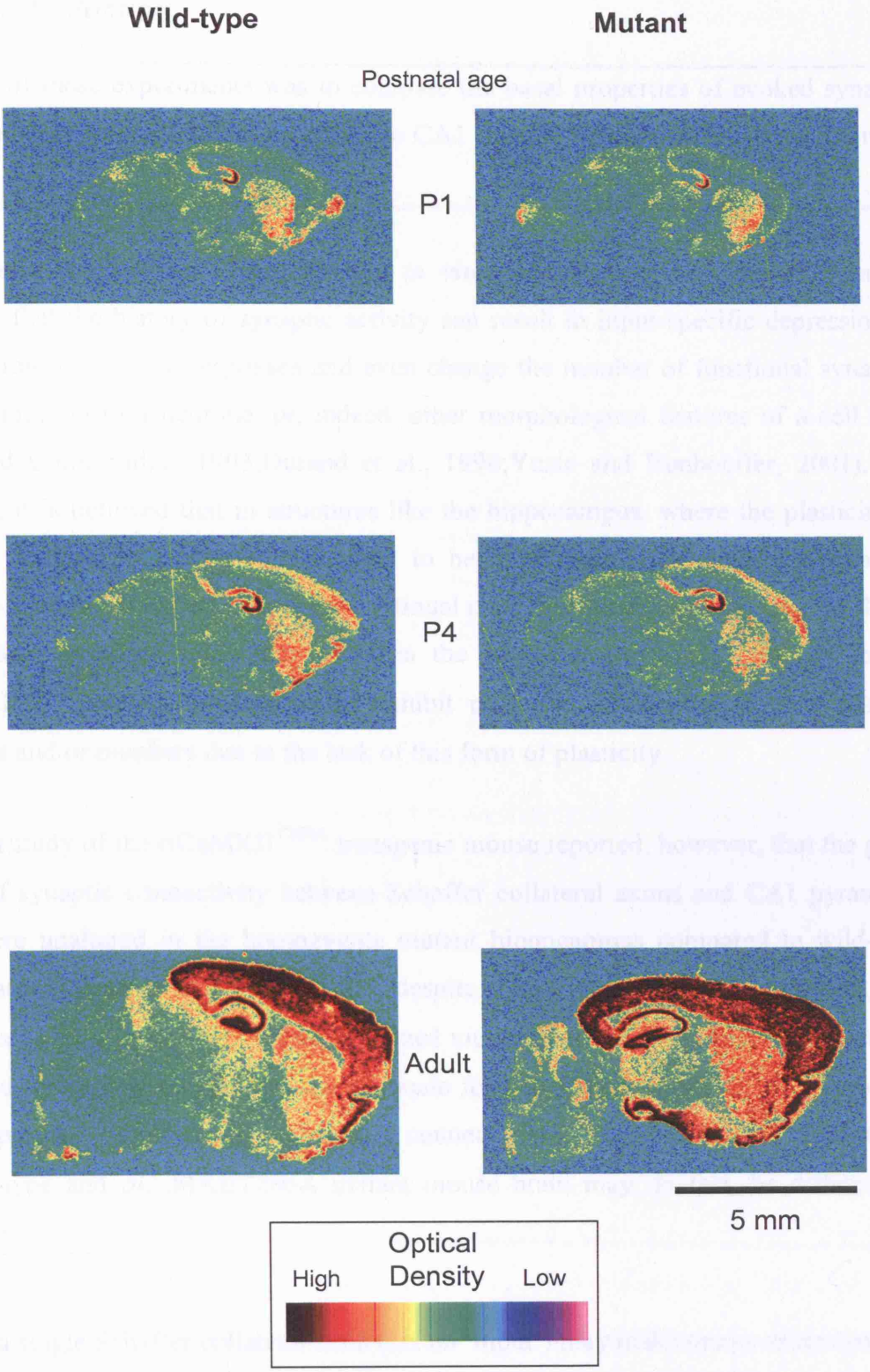
There are no detectable differences between the staining patterns of αCaMKII mRNA at all the postnatal ages studied. It must be noted, however, that saturation of the hybridization signal in the CA2/3 hippocampal cell layers from birth means that it is not possible to make conclusions about any age-dependent changes or differences between the genotypes in mRNA expression levels in these areas. This also applies to the CA1 region, the dentate gyrus and the outer layers of the cortex from P8 onwards. Despite this, it can be seen that increases in expression within CA1, the dentate gyrus and the cerebral cortex between P1 and P4 are indistinguishable between genotypes. This does suggest that the developmental control of mRNA expression is unaffected by genetic manipulation in the αCaMKIIT286A mutant mouse.

Due to the technical difficulties of high background staining, it was decided not to extend the immunocytochemical analysis to the mutant mice; however, as mRNA distributions are similar it is reasonable to assume that the distribution of protein would be similarly comparable.

Figure 3.5 The Expression Pattern of αCaMKII mRNA is not altered in the αCaMKII^{T286A} Mutant Mouse.

Example wild-type and mutant mouse brain sections show the distribution of αCaMKII mRNA at postnatal day 1 (P1), P4, and P38 (adult).

The pseudo-colour scale indicates optical density and correlates with the intensity of mRNA labelling.


Wild-type
Mutant
Postnatal age
P1
P4
Adult
5 mm
Optical Density
High
Low

3.2 Synaptic Transmission at Schaffer Collateral to CA1 Synapses in Wild-Type and Mutant Adult Mice

3.2.1 Introduction

The aim of these experiments was to compare the basal properties of evoked synaptic transmission at single Schaffer collateral to CA1 inputs* between wild-type and mutant mice.

The current mechanistic theory for the *in vivo* modification of synaptic strengths proposes that the history of synaptic activity can result in input-specific depression or potentiation of synaptic responses and even change the number of functional synapses that impinge on to a neurone, or, indeed, other morphological features of a cell (e.g. Bliss and Collingridge, 1993;Durand et al., 1996;Yuste and Bonhoeffer, 2001). For instance, it is believed that in structures like the hippocampus, where the plasticity of synaptic strength and number is thought to be fundamental for memory formation, experience and learning will cause the continual modification of neuronal circuits. Thus, if CaMKII-dependent LTP does occur in the mouse *in vivo*, the synapses in the $\alpha CaMKII^{T286A}$ mutant animals might exhibit profound differences in their relative strengths and/or numbers due to the lack of this form of plasticity.

The first study of the $\alpha CaMKII^{T286A}$ transgenic mouse reported, however, that the gross levels of synaptic connectivity between Schaffer collateral axons and CA1 pyramidal cells were unaltered in the homozygote mutant hippocampus compared to wild-type litter-mates (Giese et al., 1998). Thus, despite their finding that the summed CA1 responses arising from many inputs remained unaltered in the αCaMKIIT286A mutant (assessed using field recordings) the rationale for the investigations of this thesis was based upon the hypothesis that excitatory connectivity at the level of individual inputs in wild-type and αCaMKIIT286A mutant mouse brain may, in fact, be different. If

* Note, a single Schaffer collateral axon (i.e. an 'input') may make one or more synaptic connections with a single CA1 cell.

differences do exist, identifying such characteristics will facilitate the understanding of the endogenous forms of synaptic plasticity that occur within the CA1 region of the hippocampus.

The ideal approach for studying individual inputs would be to make paired recordings between single CA3 and CA1 neurones. This would ensure that the responses recorded in CA1 neurones arose from the stimulation of a single axon. However, the probability of finding monosynaptically coupled pyramidal cells in an acute hippocampal slice is extremely low (Sayer et al., 1990;Thomson and Radpour, 1991), making this technique highly inefficient to exploit. We therefore chose the method of 'minimal' extracellular stimulation to trigger action potentials in Schaffer collateral axons and recorded the postsynaptic responses in CA1 pyramidal neurones using the whole-cell voltage-clamp technique. Although we cannot confirm that only a single axon is being stimulated, by performing all experiments and data analysis blind to genotype and by applying the same methodology for the determination of minimal stimulation across all experiments, the comparison across animal groups is valid. A schematic representation of the hippocampal slice and the positioning of electrodes is shown in figure 3.6 A.

Minimal stimulation was thus used to evoke synaptic responses that arose from a single (or at most a few) Schaffer collateral axon(s) in order to investigate the synaptic properties in wild-type and mutant hippocampi on a much smaller level than previously assessed using field recordings (Giese et al., 1998). A paired-pulse stimulation paradigm was used.

A measure of each input's baseline level of synaptic efficacy or 'strength', reflecting both pre- and post-synaptic properties, was assessed by analysing the amplitude of the averaged response (including failures) of the first of each pair of evoked responses (i.e. EPSC1) and by calculating the probability of evoked failures for that input. The probability of failures gives a measure of presynaptic release probability. Furthermore, the paired-pulse ratio (PPR) of the averaged response from each cell was calculated which gave a measure of the input's short-term plastic properties. The PPR was calculated by dividing the peak amplitude of the second averaged EPSC by that of the first (i.e. PPR = EPSC2 / EPSC1). Hippocampal CA3-CA1 synapses exhibit paired-pulse facilitation (PPF) when two action potentials invade a pre-synaptic terminal in

close succession (10-300 ms; e.g. Dobrunz et al., 1997;Maruki et al., 2001;Oertner et al., 2002). Although the mechanisms of PPF are still not fully understood, a wealth of evidence supports the widely accepted 'residual calcium hypothesis' that was first proposed by Katz and Miledi in 1968 as the underlying cause (reviewed in Zucker and Regehr, 2002). This proposes that after the first presynaptic action potential residual calcium within the synaptic bouton will persist and result (on average) in increased neurotransmitter release in response to the second action. The extent of facilitation is also highly correlated with the initial release probability (Debanne et al., 1996;Dobrunz and Stevens, 1997;i.e. of the first stimulus; e.g. Murthy et al., 1997) and can also be used, therefore, as a measure of presynaptic efficacy.

This is the first time that pre-synaptic properties of Schaffer collateral synapses and short-term plasticity have been made in the αCaMKII^{T286A} mutant mouse.

3.2.2 *The Effects of the αCaMKII^{T286A} Mutation upon the Properties of Minimally Stimulated EPSCs*

All experiments and subsequent analyses were performed blind to genotype. Excitatory postsynaptic currents (EPSCs) were minimally evoked by electrically stimulating the axons lying within the stratum radiatum of acute hippocampal slices and responses recorded from voltage-clamped CA1 pyramidal neurones (holding potential, -70 mV). Paired stimuli (inter-stimulus interval (ISI) 70ms) were delivered every 5 seconds using a microelectrode filled with 1 M NaCl. The slices were prepared from adult (6-8 week old) αCaMKIIT286A mutant mice and their wild-type littermates. All responses were averaged, including those where no postsynaptic currents were observed (so-called 'synaptic failures' – caused by the failure of an action potential to initiate the release of a vesicle of neurotransmitter). It was assumed that each extracellular stimulus faithfully triggers the identified Schaffer collateral axon(s) to fire. All experiments were performed in the presence of 6 μM SR95331 to prevent spontaneous and evoked $GABA_A$ receptor-mediated synaptic currents from interfering with the EPSC recordings.

An example input/output plot of stimulation intensity (V) versus the averaged whole-cell current responses (pA) – used for the selection of an appropriate minimal stimulation voltage – is shown in figure 3.6 B. (The experimental procedure is described

in methods section 2.6.6.2). The averaged current traces from which these amplitude values were taken are also shown (figure 3.6 C).

For each recording, baseline levels of synaptic transmission were assessed by determining the averaged EPSC1 amplitude (including failures), the probability of EPSC1 failures and the PPR. In summary, no differences in the baseline levels of synaptic transmission were found between wild-type and mutant mice. This finding opposes our hypothesis that profound differences in synaptic transmission at individual inputs would be present despite the summed responses of many inputs being unaltered in the mutant.

All cells from both genotypes exhibited paired-pulse facilitation (PPF; i.e. PPR > 1). Figure 3.7 shows raw current traces of minimally evoked EPSCs recorded from a representative wild-type (figure 3.7 A) and mutant (figure 3.7 B) CA1 pyramidal cell. A trace containing a spontaneous synaptic current is shown. The similarity between the amplitudes of the spontaneous and the evoked EPSCs strengthens the evidence that extracellular stimulation in the stratum radiatum of this slice was probably evoking action potentials in a single connected Schaffer collateral axon. The averaged current responses of all the minimal stimuli delivered to each of these cells are also shown. The averaged currents clearly demonstrate the PPF phenomenon.

The mean amplitude (± s.e.m.) of the averaged EPSC1 (including failures) was 3.60 ± 1.65 pA (n = 7) in wild-type animals and 2.60 ± 0.74 pA (n = 14) in mutant animals (figure 3.8 A). Although these data show a trend for the EPSC1 amplitude to be smaller in the mutant cells, the difference did not reach a statistical level of significance (P = 0.0715; two-tailed t-test). A presynaptic basis for this observation was not supported by the presence of trends in either the mean probability of EPSC1 failures or the mean PRR (figures 3.8 B and C). However, we can not exclude the possibility that the trend reflects small differences in postsynaptic properties of wild-type and mutant CA1 neurones.

To test whether the scatter of the data points were different between the two genotypes, F tests were performed. Significant differences between the variances of the mean data might indicate that the extent of the range of synaptic strengths was affected by the

lack of CaMKII T286-autophosphorylation. For example, a larger range of synaptic strengths might be expected to exist in wild-type brains (where all plastic processes are intact) compared to mutant brains where a form of synaptic potentiation is lost.

The variance of EPSC1 averaged amplitudes was significantly smaller in the in wild-type animals (variance = 0.025) than in mutant animals (variance = 0.094; P = 0.0155). However, this trend was found to go in the opposite direction to that asmight be expected (i.e. that the range of synaptic efficacies might be greater in wild-type animals where synaptic potentiation is intact). There were no significant differences between variances for either the probability of failures (P = 0.7766) or the PPR (P = 0.1122). This again supports the conclusion that no presynaptic differences exist between the wild-type and mutant animals. Thus, if the differences between genotypes in the mean and variation of EPSC1 amplitudes are real, the postsynaptic membrane is the most likely locus for these dissimilarities.

Figure 3.6 Determination of Minimal Stimulation Voltage.

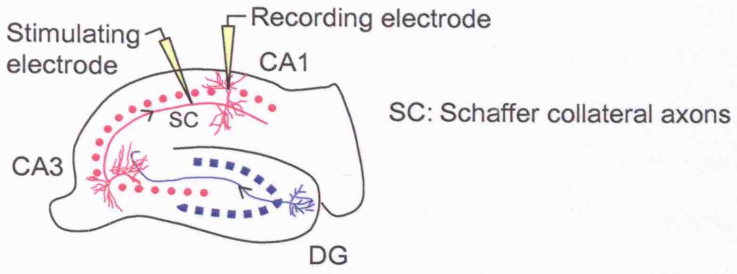
Paired electrical stimuli (ISI 70ms) were delivered every 5 seconds using a microelectrode positioned within the stratum radiatum of an acute hippocampal slice. Once a synaptic input was found, the stimulating voltage was returned to a sub-threshold intensity and then gradually increased. At each stimulation intensity (V) 20 paired stimuli were delivered. The responses were averaged (including synaptic failures) and the peak amplitudes of the two synaptic currents measured in order to select the appropriate stimulation intensity for the stimulation of a single (or at most a few) axon(s).

A) A schematic diagram of a hippocampal slice showing the relative positioning of the recording and stimulating microelectrodes. The hippocampal CA1 and CA3 sub-regions and the dentate gyrus (DG) are labelled.

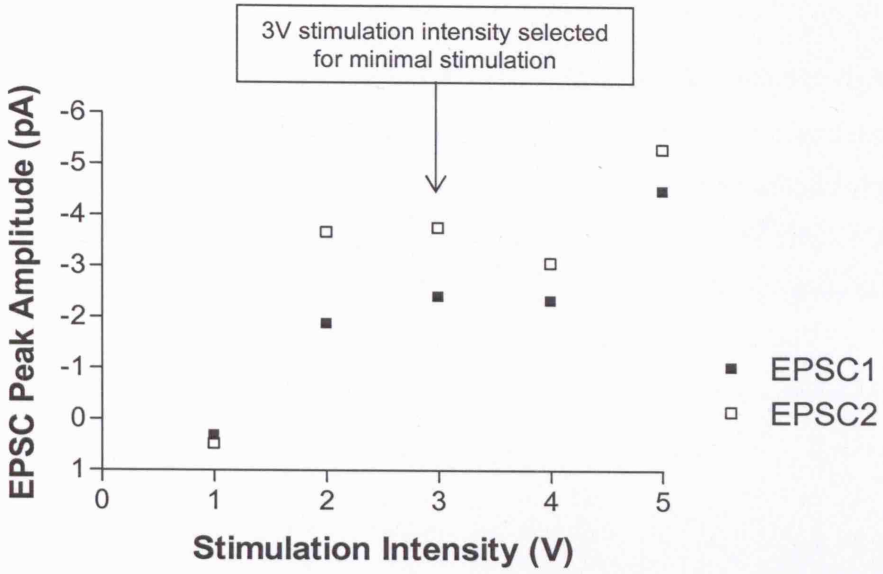
B) An example input-output plot of stimulation intensity versus EPSC peak amplitude. At the 1 V stimulation intensity no synaptic responses could be elicited. At 2, 3 and 4 Vs the EPSC1 peak amplitudes and the degree of paired pulse facilitation are similar; it is likely that the responses seen at these voltages were evoked from the stimulation of the same axon(s). At 5 V the peak amplitude of EPSC1 has approximately doubled indicating that another axon had been recruited.

C) The averaged current traces for each stimulation intensity.

A)


Stimulating electrode
Recording electrode
CA1
SC
CA3
DG
SC: Schaffer collateral axons

B)


3V stimulation intensity selected for minimal stimulation
EPSC Peak Amplitude (pA)
-6
-5
-4
-3
-2
-1
0
1
0
1
2
3
4
5
Stimulation Intensity (V)
EPSC1
EPSC2

C)

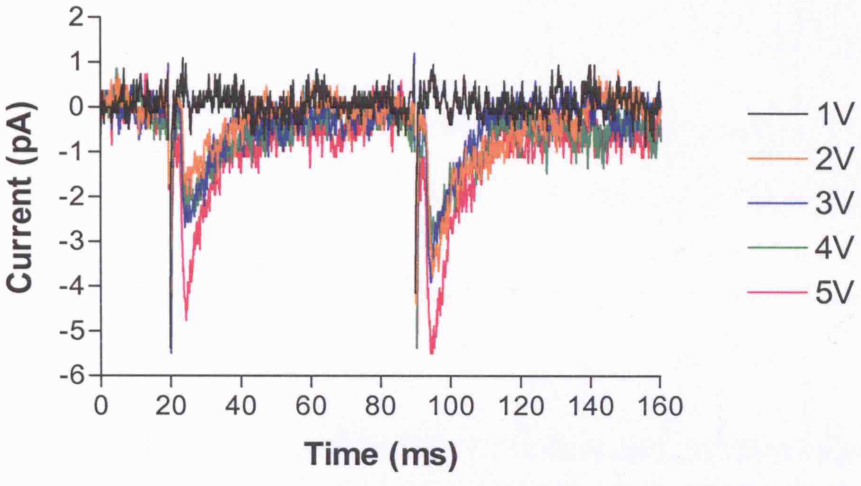

Current (pA)
2
1
0
-1
-2
-3
-4
-5
-6
0
20
40
60
80
100
120
140
160
Time (ms)
1V
2V
3V
4V
5V

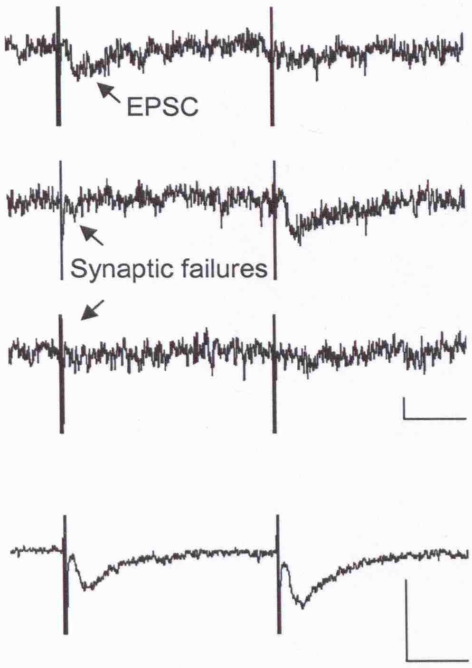
Figure 3.7 Synaptic Currents Recorded in Wild-Type and Mutant CA1 Pyramidal Neurones Evoked by Minimal Paired-Pulse Stimulation of Schaffer Collateral Axons

A) Example current traces of minimally evoked EPSCs recorded in a wild-type CA1 neurone. In the first of the three individual traces, a synaptic current can be seen to follow the stimulation artefact of the first stimulus after a short delay; the second of the paired stimuli failed to result in the synaptic transmission. The averaged current responses from all stimuli delivered to this cell at the minimal stimulation intensity are shown below. PPF is clearly evident.

B) Example current traces of minimally evoked EPSCs recorded in a mutant neurone. The asterisk (*) labels a spontaneous synaptic event. The similarity in the amplitude of the evoked EPSCs and the spontaneous EPSC support the proposal that the evoked EPSCs came from the stimulation of a single axon. Once more, the averaged current responses from all minimal stimuli are shown below.

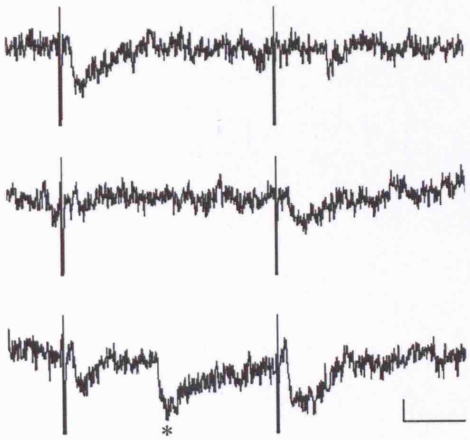
All scale bars: 5 pA, 20 ms.

A) Wild-Type



Average of n=110 consecutive stimulation sweeps

B) Mutant



Average of n=84 consecutive stimulation sweeps

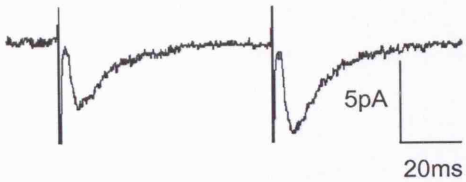


Figure 3.8 Properties of Synaptic Transmission in CA1 Pyramidal Cells Evoked by Minimal Paired-Pulse Stimulation of Schaffer Collateral Axons are unchanged in the αCaMKII^{T286A} Transgenic Mice.

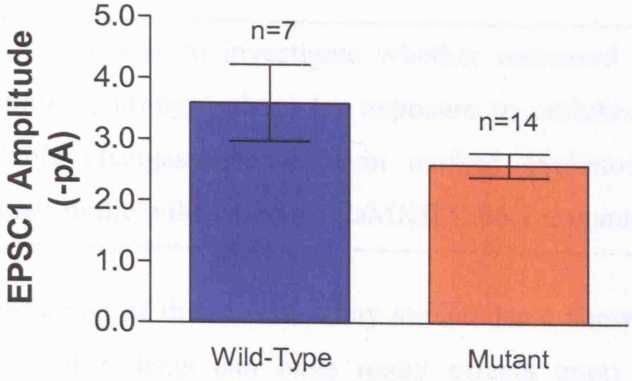
Bar graphs show means ± s.e.m.; n = number of cells. All P values were determined using unpaired two-way t-tests.

A) Mean averaged EPSC1 amplitudes (including failures). Although the difference between the genotype means did not reach significance (P = 0.072), there is a trend for the averaged EPSC1 to be smaller in the mutant than in the wild-type cells.

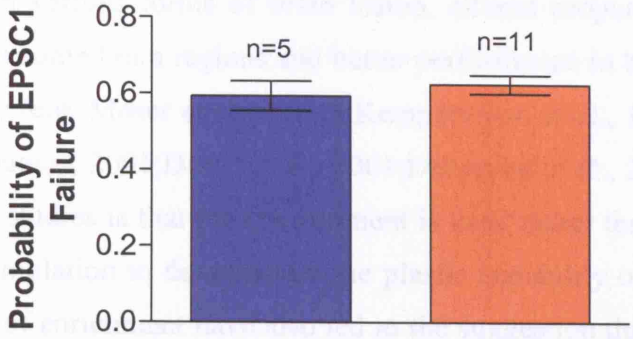
B) The mean probabilities for failures at Schaffer collateral to CA1 excitatory synapses are similar between wild-type and mutant cells (P = 0.498).

C) The mean PPR of minimally evoked EPSCs (inter-stimulus interval 70ms) did not significantly differ between wild-type and mutant cells (P = 0.314).

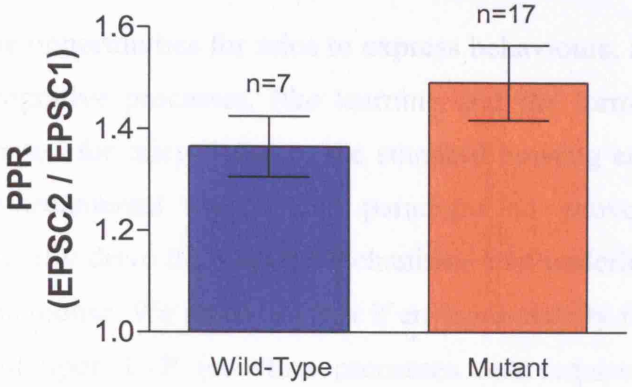
A)


EPSC1 Amplitude (-pA)
5.0
4.0
3.0
2.0
1.0
0.0
n=7
n=14
Wild-Type
Mutant

B)


Probability of EPSC1 Failure
0.8
0.6
0.4
0.2
0.0
n=5
n=11

C)


PPR (EPSC2 / EPSC1)
1.6
1.4
1.2
1.0
n=7
n=17
Wild-Type
Mutant

3.3 Synaptic Transmission in Wild-Type and Mutant Mice Raised in Enriched Housing Environments

3.3.1 Introduction

The aim of these experiments was to investigate whether increased exploration and spatial learning in the mouse, brought about by exposure to enriched environments, would result in detectable changes/differences in evoked excitatory responses at Schaffer collateral synapses in the wild-type and CaMKIIT286A mutant mice.

As reviewed in the introduction of this thesis, many studies have shown that enriching the housing environment of rodents can have many effects upon brain anatomy, neurochemistry, synaptic physiology and animal behaviour. Effects include: changes in dendritic spine densities, the increased survival of new cells in the dentate gyrus, enhanced recovery from various forms of brain lesion, altered properties of synaptic transmission and LTP in some brain regions and better performance in behavioural tests of learning and memory (e.g. Moser et al., 1994b;Kempermann et al., 1997;Rampon et al., 2000b;Foster and Dumas, 2001;Duffy et al., 2001;Dahlqvist et al., 2004). The most elegant feature of these studies is that the environment is used rather than any chemical or electrical form of stimulation to demonstrate the plastic capability of the brain. The many beneficial effects of enrichment have also led to the suggestion that the 'standard' housing condition could in fact be more accurately described as an 'impoverished' one (e.g. Van Praag et al., 2000).

We hypothesised that the opportunities for mice to express behaviours, like exploration, and to recruit higher cognitive processes, like learning and the formation of spatial memories, would be limited for mice living in the standard housing environment. We therefore used an environmental enrichment paradigm to provoke explorative behaviours and to potentially drive the plastic mechanisms that underlie hippocampal-dependent learning in the mouse. We predicted that if environmentally-induced synaptic changes were dependent upon LTP (or other processes that require the actions of autophosphorylated αCaMKII) they would not be seen in the mutant mice. Furthermore, if enrichment is found to uncover a difference in synaptic connectivity between the wild-type and αCaMKIIT286A mice, the observed lack of differences between the two

genotypes raised in standard housing conditions could in fact be considered as a pathological artefact due to effective environmental 'impoverishment'.

3.3.2 *The Effects of Environmental Enrichment upon the Properties of Minimally Stimulated EPSCs*

Wild-type and mutant littermates were housed for 3-5 weeks, post weaning, in enriched environments; an example of the enriched housing condition is shown in figure 2.2. Table 3.1 presents summarised data for electrophysiological experiments performed in the four experimental animal groups.

The bar charts in figures 3.9 A-C respectively show the mean values (± s.e.m.; n cells) of: a) EPSC1 averaged amplitudes (including failures), b) probability of EPSC1 failures and c) PPR, for wild-type and mutant hippocampal slice CA1 recordings obtained from animals raised in both standard and enriched housing conditions. The data shown for recordings from mice raised in standard conditions are the same as those represented in figure 3.8. Experiments upon mice from standard and enriched environments were performed contemporarily. This excludes the possibility that any effects of environment arose from seasonal influences. The mean of the EPSC1 decay time constants, for each group are also presented (figure 3.9 D).

To determine how synaptic properties were affected by environment and genotype, analysis of variance (ANOVA) was used followed by Bonferroni post-tests. The overall null hypothesis for interaction between genotype and housing condition states that any effects of environmental enrichment upon the properties of synaptic transmission are independent of animal genotype. The null hypotheses for the individual effects of enrichment and genotype respectively state that any differences between enriched animals (independent of genotype) and any differences between wild-types and mutants (independent of housing condition) are due to chance. When an overall significant effect of enrichment was observed, Bonferroni post-tests were used to specifically ask whether the effects were restricted to either the wild-type or the mutant animals.

The surprising result was that these data show that the environmental enrichment paradigm primarily affected minimally evoked EPSC responses in the mutant animals,

whilst synaptic responses obtained from wild-type CA1 neurones where largely unaffected.

There was a significant interaction between genotype and housing condition upon the mean amplitude of minimally evoked EPSC responses (including failures; $P = 0.0196$), indicating that post-weaning development in enriched environments did not result in similar effects in wild-type and mutant brains. The Bonferroni post-tests indicated that enrichment significantly altered synaptic efficacy in mutant ($P < 0.05$) but not in the wild-type CA1 neurones ($P > 0.05$). The mean amplitude of unitary responses was 57% higher in mutant mice raised in enriched environments than mutants raised in standard conditions (figure 3.9 A).

The analysis of both the probability of failures (figure 3.9 B) and the PPRs (figure 3.9 C) both support the view that the observed increase in EPSC1 amplitudes in the enriched mutant mice is due to higher presynaptic release probabilities.

With regards to the probability of failures, interaction between genotype and housing condition did not quite reach significance ($P = 0.0650$), thus, we can not confidently conclude that the effects of enrichment were different in wild-type and mutant mice. Genotype alone did not significantly affect the probability of failures ($P = 0.1101$), whereas, the type of housing condition did ($P = 0.0034$). The Bonferroni post-tests revealed that the effects of enrichment upon failure rate were again only significant in the mutant animals ($P < 0.01$) and were not in the wild-types ($P > 0.05$). Thus, despite no significant interaction, the effect of enrichment to lower synaptic vesicle release probability was greater in mutant mice (29%, compared to 4% in wild-types).

Note, a lower probability of evoked failures might also arise from a postsynaptic difference in the enriched mutants. For example, if the Schaffer collateral terminals contain more than one release site, the decrease in failure rate could also reflect a decrease in the incidence of postsynaptic silent synapses, although by itself this would not be expected to be accompanied by a change in the paired-pulse ratio.

The mean level of paired-pulse facilitation was significantly lower in mutant mice exposed to environmental enrichment (figure 3.9 C). There was significant interaction

between housing condition and genotype ($P = 0.0295$) and Bonferroni post-tests indicated that enrichment only affected PPF in mutant synapses ($P < 0.01$). In the mutants, the mean level of PPF was 59% lower in animals raised in enriched environments compared to those raised under standard conditions. In wild-types, PPR was largely unaffected by environment (PPF was 2% higher in enriched compared to standard animals).

The EPSC1 decay time-constant was also determined for each recording and the mean values (± s.e.m.; n cells) for each animal group are presented in figure 3.9 D. The reason why decay time-constants were important to assess is due to the fact that the peak amplitude for both current events were measured with respects to a zero current level determined before the stimulation artifact of the EPSC1. Longer EPSC1 decay times in one animal group could artificially increase the measured PPF values by an effect of EPSC2 summation upon the tail of the ESPC1 decay. Decay kinetics were not affected by animal genotype or housing environment nor did these two factors show any interaction. Thus the differences in PPR could not be accounted for, or biased by, differences in the decay properties of the minimally evoked EPSCs.

In summary, enrichment of the environment leads to changes in the basal properties of Schaffer collateral to CA1 synaptic transmission in αCaMKIIT286A mutant mice whilst wild-type synapses appear unaffected. The lower probability of failures and higher levels of paired pulse facilitation suggest that the enrichment increases release probability in the mutant animals. These changes may also underlie the observed increase in mean EPSC1 amplitude although other postsynaptic changes may also have occurred and differences in the number of silent synapses can not be excluded. These summary findings are represented in figure 3.10. The example traces of recordings from mutant neurones clearly show that paired-pulse synaptic responses facilitate less in CA1 recordings from mice raised in environmentally enriched conditions (figures 3.10 B).

Figure 3.9 Environmental Enrichment alters Pre-Synaptic Properties of Schaffer Collateral Inputs that Impinge onto CA1 Pyramidal Cells in $\alpha CaMKII^{T286A}$ Mutant Mice.

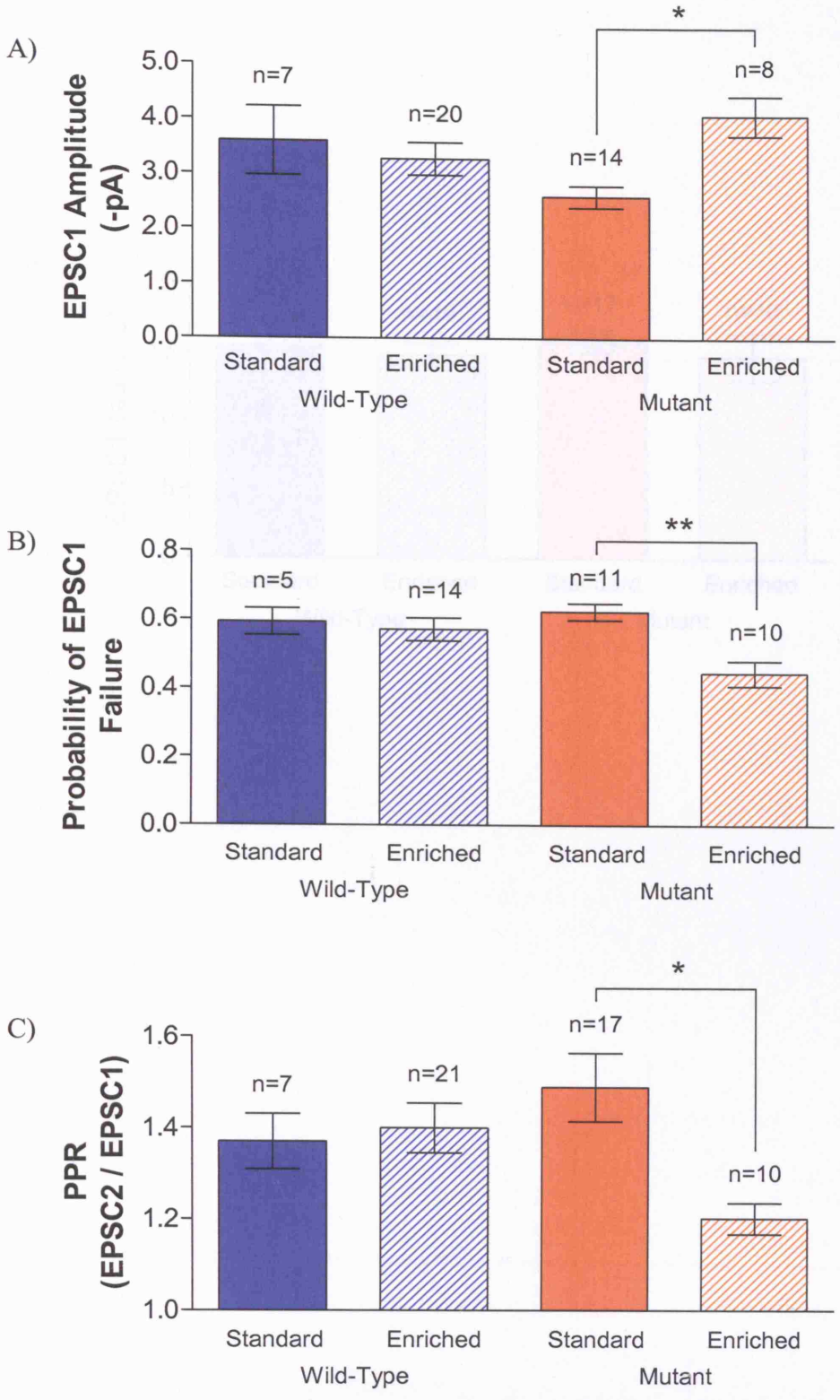
Bar graphs show means ± s.e.m.; n = number of cells. Values from the four experimental animal groups were statistically analysed using two-way ANOVA and Bonferroni post-tests; asterisks refer to Bonferroni post-tests: * $P<0.05$, ** $P<0.01$.

A) Mean averaged EPSC1 peak amplitudes are increased in $\alpha CaMKII^{T286A}$ mutant CA1 neurones from mice raised in environmentally enriched conditions. There is a significant interaction between genotype and housing condition upon the averaged amplitudes of minimally stimulated EPSCs ($P = 0.0196$). Bonferroni post-tests show that the effects of enrichment are significant in recordings from $\alpha CaMKII^{T286A}$ cells ($P < 0.05$), but insignificant in recordings from wild-type cells ($P > 0.05$).

B) Mean probability of failures. The interaction between genotype and housing condition upon the probability of EPSC1 failures does not quite reach significance ($P = 0.065$); however, variation in the data is significantly affected by housing condition ($P = 0.0034$) albeit unaffected by genotype ($P = 0.11$). Bonferroni post-tests show that the effects of enrichment reach significant levels in the mutant cells ($P < 0.01$), but not in the wild-type recordings ($P > 0.05$).

C) Mean PPRs. There is a significant interaction between genotype and housing condition upon the PPR of minimally stimulated EPSCs ($P = 0.0295$). Bonferroni post-tests again indicated a significant effect of enrichment in the mutant cells ($P < 0.05$), but not in wild-type recordings ($P > 0.05$).

D) EPSC1 decay time constants are unaffected by animal group. (Interaction, $P = 0.89$; effect of genotype, $P = 0.95$; effect of housing condition $P = 0.57$).


A)
EPSC1 Amplitude (-pA)
5.0
4.0
3.0
2.0
1.0
0.0
n=7
n=20
n=14
n=8
*
Standard
Enriched
Standard
Enriched
Wild-Type
Mutant
B)
Probability of EPSC1 Failure
0.8
0.6
0.4
0.2
0.0
n=5
n=14
n=11
n=10
**
Standard
Enriched
Standard
Enriched
Wild-Type
Mutant
C)
PPR (EPSC2 / EPSC1)
1.6
1.4
1.2
1.0
n=7
n=21
n=17
n=10
*
Standard
Enriched
Standard
Enriched
Wild-Type
Mutant

D)

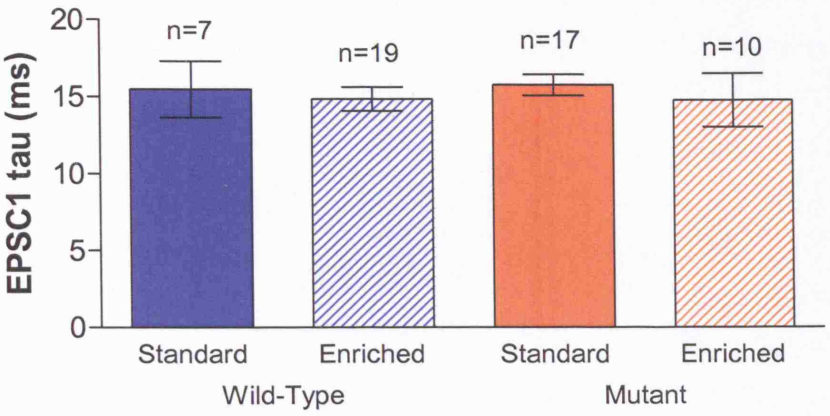
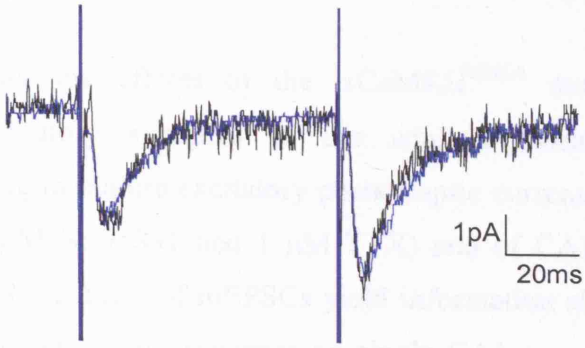

EPSC1 tau (ms)
20
15
10
5
0
n=7
n=19
n=17
n=10
Standard
Enriched
Standard
Enriched
Wild-Type
Mutant

Figure 3.10 Paired-Pulse Synaptic Responses Facilitate less in CA1 Recordings from αCaMKII^{T286A} Mutant Mice Raised in Environmentally Enriched Conditions.

Overlapping example traces of averaged paired-pulse synaptic responses recorded from CA1 neurones from: A) wild-type and B) i) αCaMKII^{T286A} mutant mice raised in standard (black trace) and environmentally enriched (blue trace) housing conditions. Responses in wild-type neurones are indistinguishable between the two housing conditions; whereas, in the mutant recordings, the EPSC1 peak amplitude is larger and the degree of facilitation less in the cell from the enriched environment.

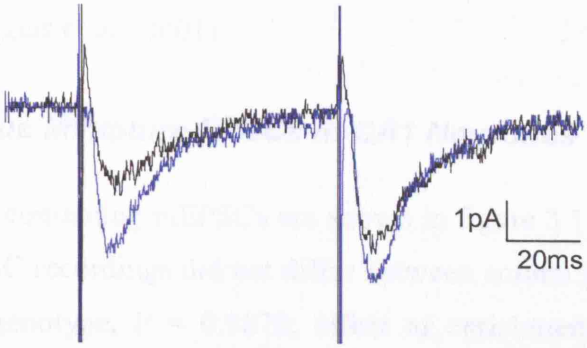
B ii) The same traces as shown in i) normalised with respects to EPSC1 peak amplitude. This figure demonstrates the smaller amount of paired-pulse facilitation seen in mutant CA1 recordings from mice raised in the enriched environment.

A) Wild-Types

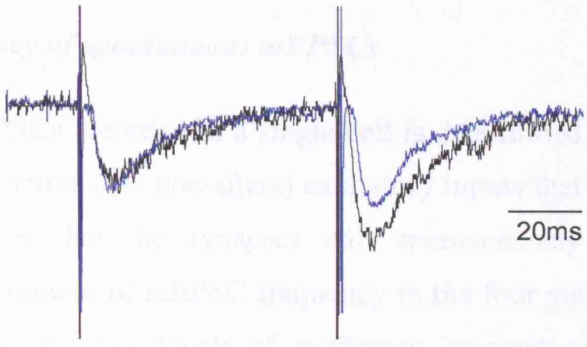


B) Mutants

i)



ii) Normalised to EPSC1 peak amplitude



3.4 Gross levels of Excitatory Connections in CA1 Pyramidal Neurones: mEPSC and Spine Density measurements

3.4.1 Introduction

To characterise further the effects of the αCaMKII^{T286A} mutation and enriched experience upon excitatory synapses in the adult hippocampus, measures of spontaneously occurring miniature excitatory postsynaptic currents (mEPSCs; recorded in the presence of 6 μM SR95331 and 1 μM TTX) and of CA1 cell dendritic spine densities were made. Recordings of mEPSCs yield information about the properties of the entire population of excitatory synapses on single CA1 neurones, whilst dendritic spine densities will largely correlate with the density of axospinous glutamatergic synapses since it has been shown that virtually all spines within the CA1 region of hippocampus receive only one synaptic contact (Harris and Stevens, 1989;Schikorski and Stevens, 1997;Megías et al., 2001).

3.4.2 Spontaneous Miniature EPSCs in CA1 Neurones

Sample current traces containing mEPSCs are shown in figure 3.11. The mean baseline noise levels for mEPSC recordings did not differ between animal groups (Interaction, P = 0.3861; effect of genotype, P = 0.9870; effect of enrichment, P = 0.7984). This indicates that the recording conditions were neither affected by genotype nor by environment – an important result since the signal-to-noise ratio is low in these recordings.

3.4.2.1 The frequency of spontaneous mEPSCs

The frequency of mEPSCs recorded in a single cell is determined by two main factors: 1) the total number of active (i.e. non-silent) excitatory inputs that it receives and 2) the underlying probabilities that the synapses will spontaneously release vesicles of neurotransmitter. Assessment of mEPSC frequency in the four animals groups gives an overall measure of the relative levels of excitatory innervation that the CA1 cells receive. Increases in synaptic release probabilities have previously been shown to correlate with increases mEPSCs frequencies (e.g. Morales et al., 2000). However, there were no significant differences between the four animal groups in the mean mEPSC

frequencies (figure 3.12 A). In fact any trend was towards a specific decrease in frequency in the enriched mutant animals. Note, the frequency of mEPSCs detected were very low in these recordings, on average no more than 1 or 2 per minute – a feature most likely related to the fact that these experiments were carried out at room temperatures.

Since the Schaffer collaterals are only one of the synaptic pathways innervating CA1 neurones, it is possible that changes in mEPSC frequency in these inputs are not large enough to be detected when assessing the total mEPSC frequency. On the other hand, differences in synapse number between the enriched and standard housing mutant groups could also account for this result.

3.4.2.2 The median amplitudes of mEPSCs

In each experiment, the peak amplitudes of all recorded mEPSCs were measured and the median value calculated; example frequency histograms of events recorded are shown in figure 3.12 D for i) a wild-type neurone from the standard housing environment and ii) a wild-type neurone from enriched environments. Median values were used as frequency histograms of CA1 unitary EPSC amplitudes are known to form skewed distributions (Bekkers and Stevens, 1989;Bekkers and Stevens, 1995;Tyler and Pozzo-Miller, 2001;Tyler and Pozzo-Miller, 2003). The mean of the median values per animal group are shown in figure 3.12 B.

Enrichment caused a small decrease in mEPSC amplitude in both genotypes: 15% in mutant neurones and 9% in wild-type neurones; there was no significant effect of genotype ($P = 0.4075$) nor any interaction between genotype and environment ($P = 0.4382$). The overall effect of enrichment alone was very significant ($P = 0.0009$), however Bonferroni post-tests indicate that the effect of enrichment only reached statistically significant levels in the mutant animals ($P < 0.01$).

Although the Bonferroni post-tests only revealed a significant effect of enrichment upon the means of the median mEPSC amplitudes in the mutant mice, the lack of any interaction between housing condition and genotype as assessed by ANOVA, and the highly significant effect of housing condition ($P = 0.0009$) prompted us to take a closer

look at these data to investigate whether the wild-type data where affected in any other way despite the mean values not showing a significant difference. Figure 3.12 E presents cumulative fraction plots of the mEPSC median amplitudes showing the relative distribution of median mEPSC amplitudes for i) wild-type and ii) mutant recording from mice raised in both standard and enriched conditions. The probabilities that standard and enriched values within each genotype were drawn from the same population were statistically tested using the Kolmogorov-Smirnov (K-S) test. For the wild-type mice, the K-S distance = 0.667 and P = 0.044; thus the null hypothesis stating that the mEPSC median amplitudes for standard and enriched wild-type mice were drawn from the same population can be rejected. It can be concluded, therefore, that although the mean of the median mEPSC amplitude across wild-type cells is not significantly affected by enrichment, the distribution of median mEPSC amplitudes across recordings was altered. Figure 3.12 E i) shows that the enrichment resulted in the median mEPSC amplitudes becoming more similar, i.e. the distribution of values is reduced. For mutant mice, the K-S distance = 0.542 and P = 0.108; in this case, enrichment did not cause a significant change to the spread of median mEPSC amplitudes across CA1 recordings.

Figure 3.12 E iii) and iv) show cumulative fraction plots for the averaged EPSC1 amplitudes for minimally evoked whole-cell currents in CA1 neurones from wild-type and mutant neurones respectively. In contract to the mEPSC data, environmental enrichment in wild-type mice does not result in a change in the distribution of EPSC1 amplitudes (K-S distrance = 0.30, P = 0.655). A significant change in the distribution was found in the mutants (K-S distrance = 0.66, P = 0.012), supporting the conclusion that these data were not drawn from the same population, as was seen with ANOVA.

Importantly, mEPSCs rise-times (the time to rise from 20% to 80% of the peak current) were not affected by enrichment (P = 0.6520, ANOVA). Thus, the enrichment induced decrease in mEPSC amplitudes recorded in the mutant mice did not arise because of an enrichment associated increase in dendritic filtering that could have attenuated mEPSC amplitude.

However, a significant effect of genotype upon rise-time was revealed (P = 0.0212) with mutant mEPSCs exhibiting faster rise-times than wild-type mEPSCs. There was no

interaction between genotype and housing condition (P = 0.3806). The simplest interpretations of these data would be that the lack of αCaMKII Thr286 autophosphorylation in mice results in the electrical shortening of the CA1 pyramidal cells and thus reduced mean levels of dendritic filtering upon unitary synaptic currents. This might result from an overall decrease in neuronal size or from a larger proportion of recorded mEPSCs arising in regions more proximal to the CA1 cell soma.

Mean rise-times (± s.e.m; n of cells) for mEPSCs recorded from wild-type neurones were 0.83 ms (± 0.08; n = 9) and 0.87 ms (± 0.09; n = 6) for mice raised in standard and enriched environments respectively; for mutant mice, the respective values were 0.71 ms (± 0.06; n = 7) and 0.61 ms (± 0.06; n = 9).

The detection of the smaller amplitude mEPSCs was difficult due to the low signal to noise ratio of these recordings. Furthermore, a selection criteria was imposed stating that all accepted events must have a peak amplitude ≥4 pA. This was to minimise the possibility that either random noise fluctuations were selected as mEPSCs or that the proportion of miniature events concealed by noise would be affected by small differences in the levels of baseline noise. As a result of this criteria however, the detection of enrichment induced decreases in mEPSC amplitude may have been limited if a larger proportion of the smaller currents were less than 4 pA and were thus being either lost in the noise or rejected. This would increase the apparent median mEPSC amplitude. The loss of smaller amplitude mEPSCs into the noise could also explain the trend towards a decrease in mEPSC frequency in the enriched mutant mouse group. Indeed, correlation analysis performed between median mEPSC amplitude and frequency shows a strong trend for a decrease in mEPSC frequency for cells with smaller amplitude events (P = 0.0514; r^2 = 0.1207; Pearson's correlation statistics; figure 3.12 C).

This effect of enrichment to decrease miniature EPSC amplitude may also indicate that the observed increases in evoked EPSC amplitude seen in the enriched mutant group does not arise from increases in postsynaptic efficacy. However, as discussed above, we can not be sure that changes in the overall median amplitudes of mEPSCs necessary reflects postsynaptic changes at Schaffer collateral synapses but it may also reflect

changes in release probability at specific types of synapse.

3.4.2.3 Correlation analyses to investigate the extent of co-variability between experimental conditions and mEPSC measurements

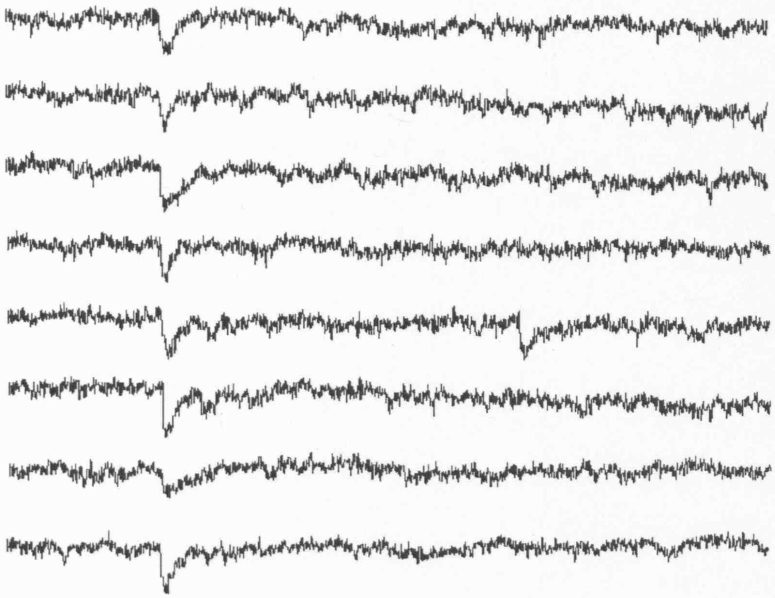
Although the mean levels of baseline noise did not differ between animal groups, two-tailed Pearson correlation tests were also performed in order to investigate the possibility that variation in experimental recording conditions (i.e. base line noise levels) could influence the detection of events and thus the apparent median amplitudes and mean frequencies of mEPSCs recorded events.

Correlation tests, performed upon data from all animal groups combined, show that the levels of baseline noise from whole-cell recordings are not correlated with mEPSC median amplitude ($P = 0.7257$; $r^2 = 0.0042$) or mEPSC frequency ($P = 0.2038$; $r^2 = 0.0533$; figures 3.13 A and B). Neither were there any significant correlations when data was assessed within each animal group (data not shown). The lack of any correlation shows that the variations in noise levels between recordings were not affecting the ability to detect mEPSCs.

Figure 3.11 Sample current traces of spontaneous mEPSCs.

Aligned sections of raw current trace showing detected mEPSCs from a (A) wild-type and (B) mutant CA1 neurone. Both recordings were made in slices obtained from mice raised in standard laboratory conditions.

A) Wild-Type



B) Mutant

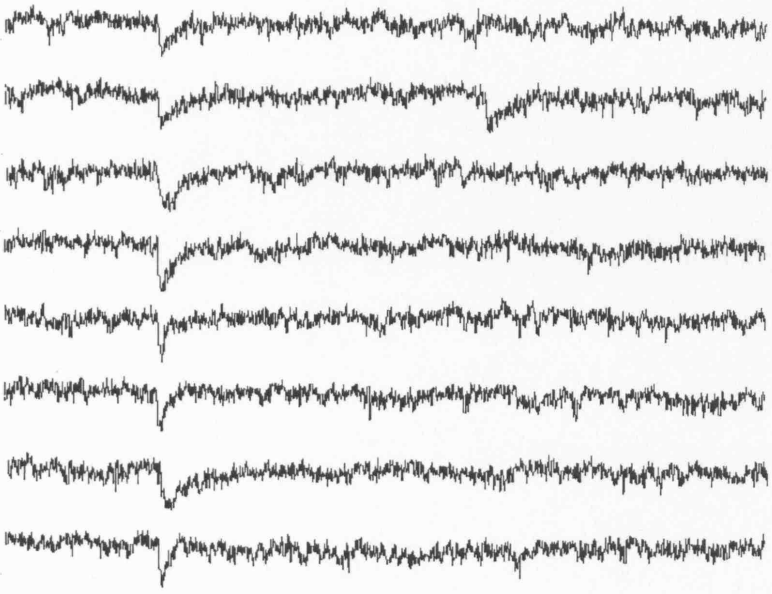


Figure 3.12 Effects of the αCaMKII^{T286A} Mutation and Environmental Enrichment upon mEPSC Frequency and Amplitude.

Bar graphs show means ± s.e.m.; n = number of cells. Statistical analysis performed with two-way ANOVA and Bonferroni post-tests; ** $P<0.01$.

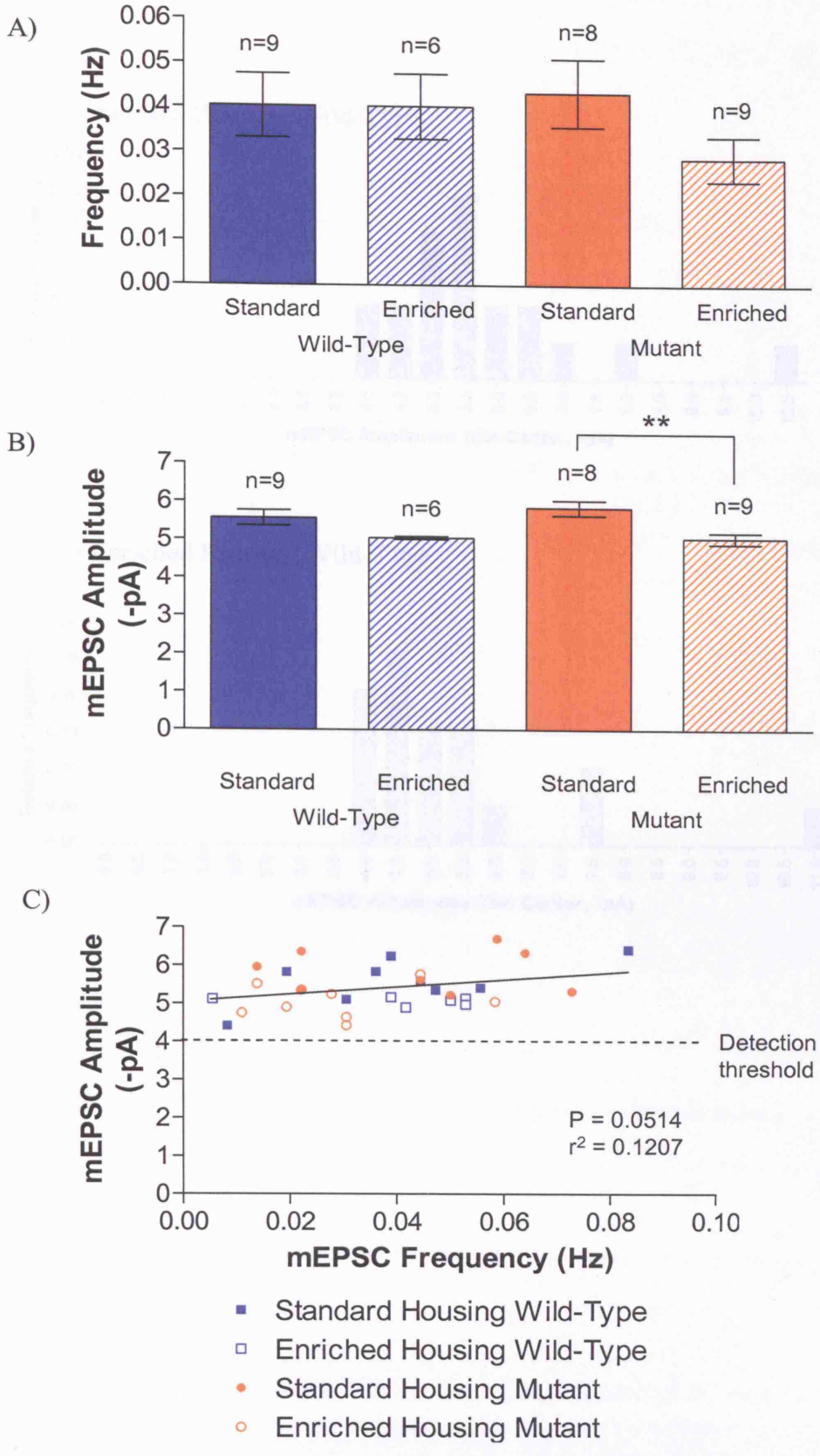
A) Mean mEPSC frequencies. No differences in mEPSC frequency were observed between groups. (Interaction, $P = 0.2986$; effect of genotype, $P = 0.2901$; effect of housing condition 0.5428).

B) Mean median mEPSC amplitudes. There was a highly significant effect of housing environment (ignoring genotype) upon median mEPSCs amplitude ($P = 0.0009$). There was neither an affect of genotype ($P = 0.4075$) nor any interaction ($P = 0.4382$) between these two variables. The enrichment-mediated decrease in mEPSC median amplitudes only reached levels of significance in the mutant recordings ($P < 0.01$; wild-types, $P > 0.05$)

C) Plot of mEPSC frequency against median amplitudes. The line of best fit and Pearson correlation statistics are shown.

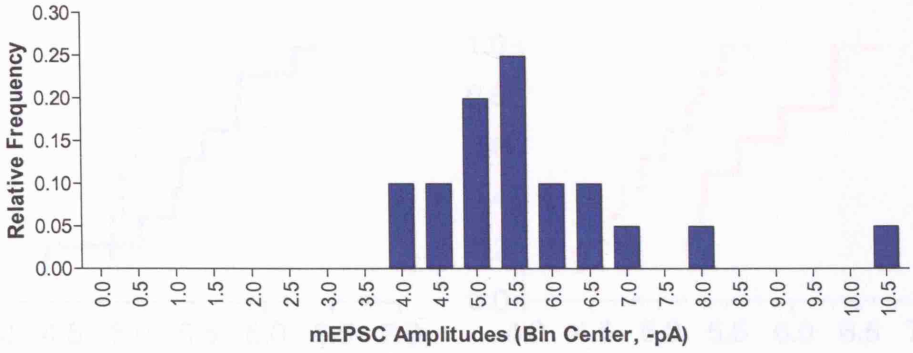
D) Example frequency histograms of mEPSC amplitudes for i) a wild-type neurone from standard housing, and ii) a wild-type neurone from enriched housing.

E) Cumulative Fraction plots for median mEPSC amplitudes from i) wild-type and ii) mutant mice, and for the averaged EPSC1 amplitudes for minimally evoked currents in iii) wild-type and iv) mutant neurones.

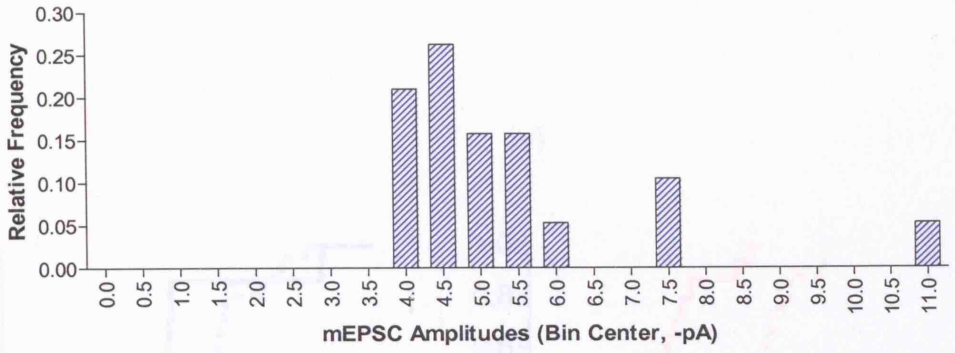

A)
Frequency (Hz)
0.06
0.05
0.04
0.03
0.02
0.01
0.00
n=9
n=6
n=8
n=9
Standard
Enriched
Standard
Enriched
Wild-Type
Mutant
B)
mEPSC Amplitude (-pA)
**
7
6
5
4
3
2
1
0
n=9
n=6
n=8
n=9
Standard
Enriched
Standard
Enriched
Wild-Type
Mutant
C)
mEPSC Amplitude (-pA)
Detection threshold
P = 0.0514
r2 = 0.1207
0.00
0.02
0.04
0.06
0.08
0.10
mEPSC Frequency (Hz)
Standard Housing Wild-Type
Enriched Housing Wild-Type
Standard Housing Mutant
Enriched Housing Mutant

D)

i) Standard Housing Wild-Type

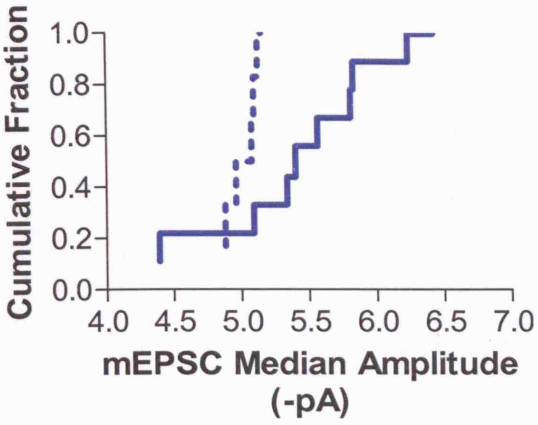


ii) Enriched Housing Wild-Type

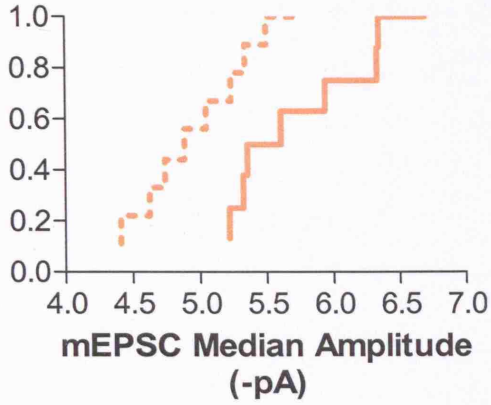


E)

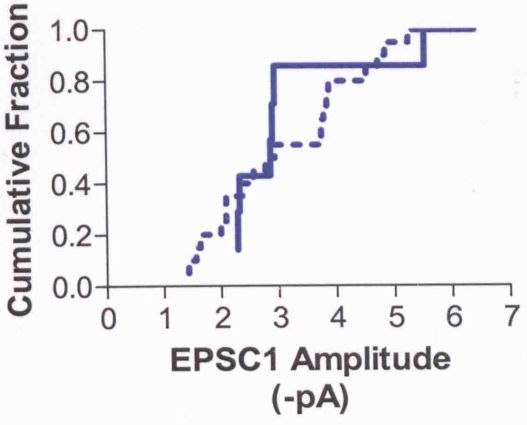
i)


Cumulative Fraction
1.0
0.8
0.6
0.4
0.2
0.0
4.0 4.5 5.0 5.5 6.0 6.5 7.0
mEPSC Median Amplitude
(-pA)

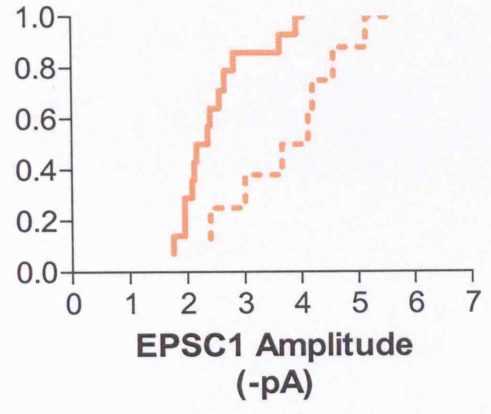
ii)


1.0
0.8
0.6
0.4
0.2
0.0
4.0 4.5 5.0 5.5 6.0 6.5 7.0
mEPSC Median Amplitude
(-pA)

iii)


Cumulative Fraction
1.0
0.8
0.6
0.4
0.2
0.0
0 1 2 3 4 5 6 7
EPSC1 Amplitude
(-pA)

iv)


1.0
0.8
0.6
0.4
0.2
0.0
0 1 2 3 4 5 6 7
EPSC1 Amplitude
(-pA)

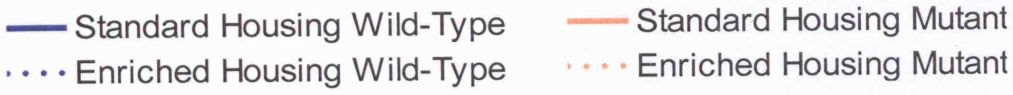
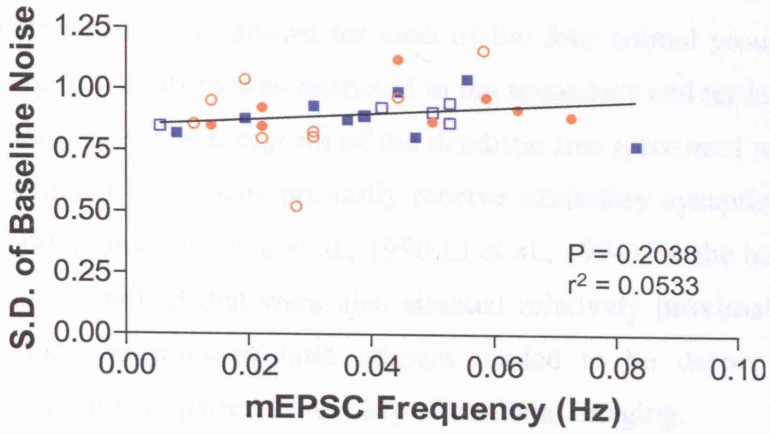

Standard Housing Wild-Type
Enriched Housing Wild-Type
Standard Housing Mutant
Enriched Housing Mutant

Figure 3.13 Baseline Noise does not Affect the Detection of mEPSCs.

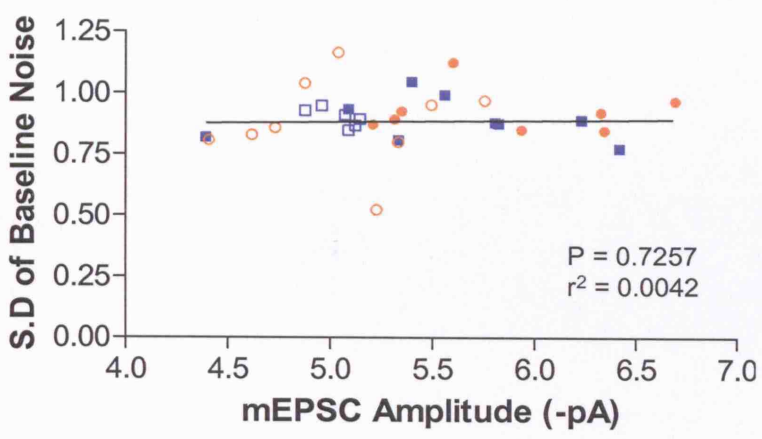
For each cell, the mEPSC frequency (A) and the median amplitude (B) is plotted against the mean standard deviation (S.D.) of the baseline noise for that recording. Each point represents a single cell.

Lines of best fit and Pearson correlation statistics are shown.

A)



B)



- Standard Housing Wild-Type
- Enriched Housing Wild-Type
- Standard Housing Mutant
- Enriched Housing Mutant

3.4.3 CA1 Pyramidal Neurone Dendritic Spine Densities

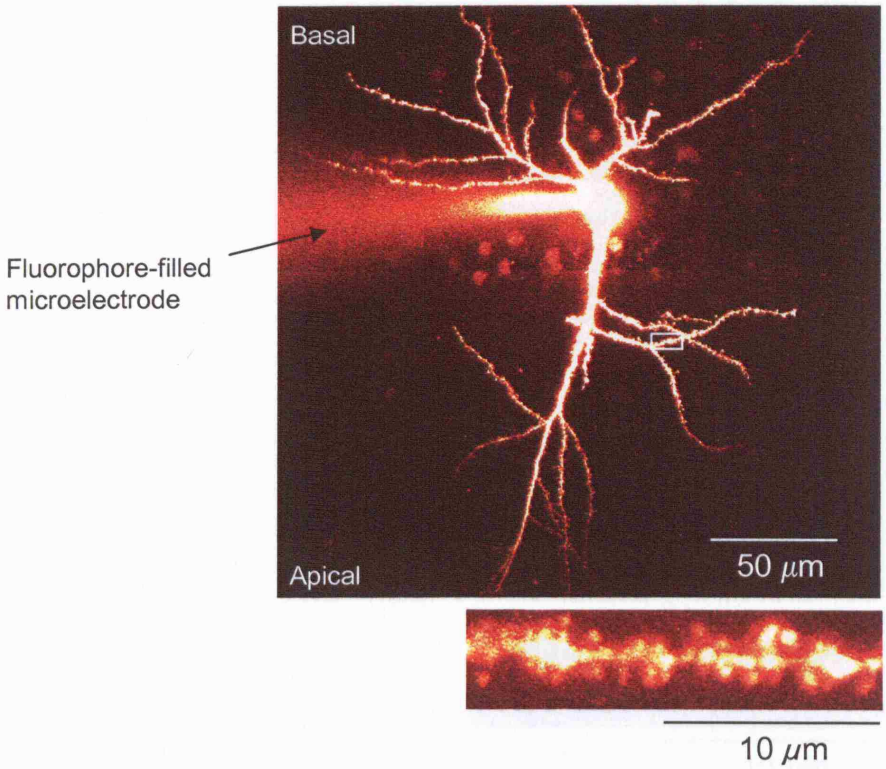
To investigate whether the αCaMKII^{T286A} mutation and/or enrichment may affect synapse number in CA1 neurones, dendritic spine densities were compared between the four animal groups. Dendrite sections within both the apical and basal dendritic arbours were analysed. Example confocal images of CA1 pyramidal neurones along with zooms of indicated dendritic sections are shown for each of the four animal groups in figure 3.14. In the apical regions, analysis was restricted to the secondary and tertiary dendrites located within the mid to proximal regions of the dendritic tree (proximal with respects to the soma). These dendritic regions primarily receive excitatory synaptic input from the Schaffer collateral axons (Ishizuka et al., 1990;Li et al., 1994). In the basal regions, dendritic sections were studied that were also situated relatively proximal to the cell soma. The more distal sections of both arbours tended to be deeper within the hippocampal slices, and this impaired the quality of confocal imaging.

Mean spine densities and the total lengths of dendritic regions studied per animal group are shown in table 3.2.

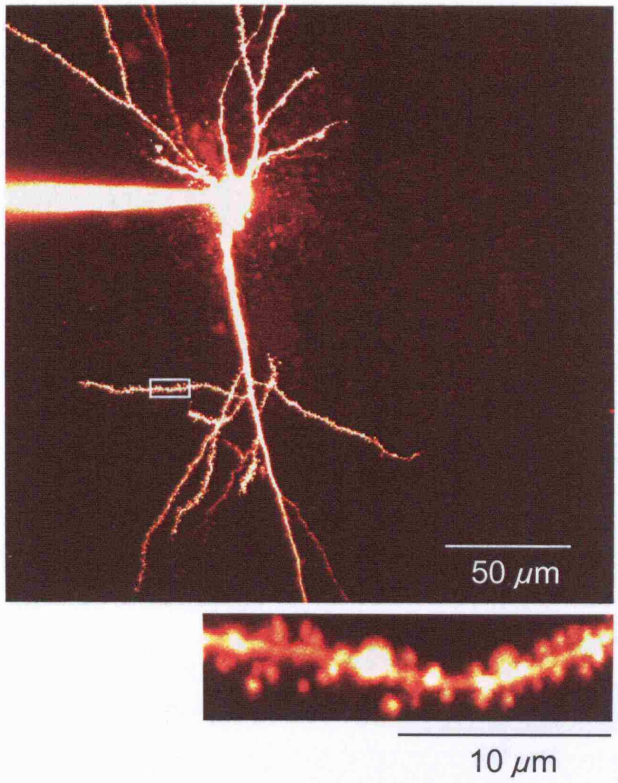
Figure 3.14 Confocal Images of Wild-Type and Mutant CA1 Pyramidal Neurones from Mice raised in Standard and Enriched Housing Environments.

Confocal images of live CA1 neurones from each of the four animal groups (A-D) show the extent of the dendritic arbour visible for analysis. The orientation of the respective basal and apical CA1 dendrites are indicated in A). An example high-zoom image of an apical dendrite section is shown for each cell; the white box indicates the location of the zoomed section within the dendritic tree. All images were taken using a 60x water-immersion objective (numerical aperture, 0.9). The flourophore-filled microelectrode is visible in each image.

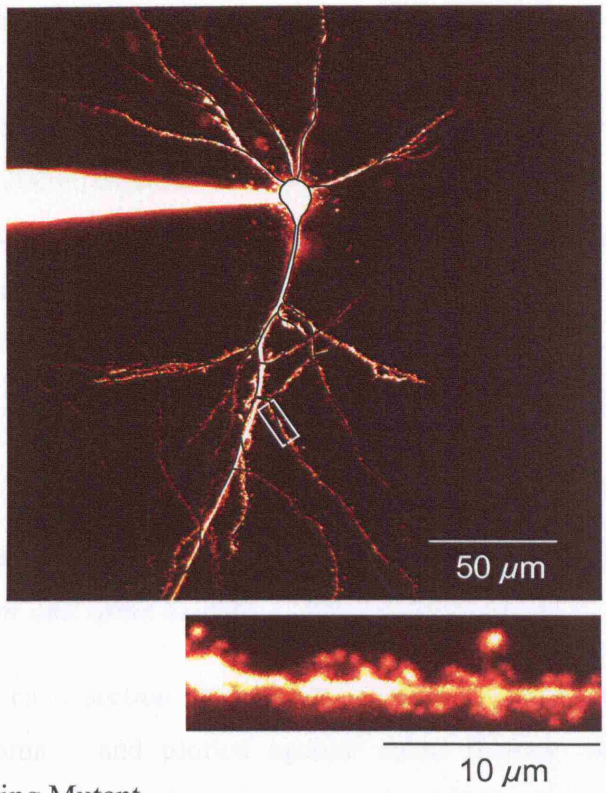
A) Standard Housing Wild-Type



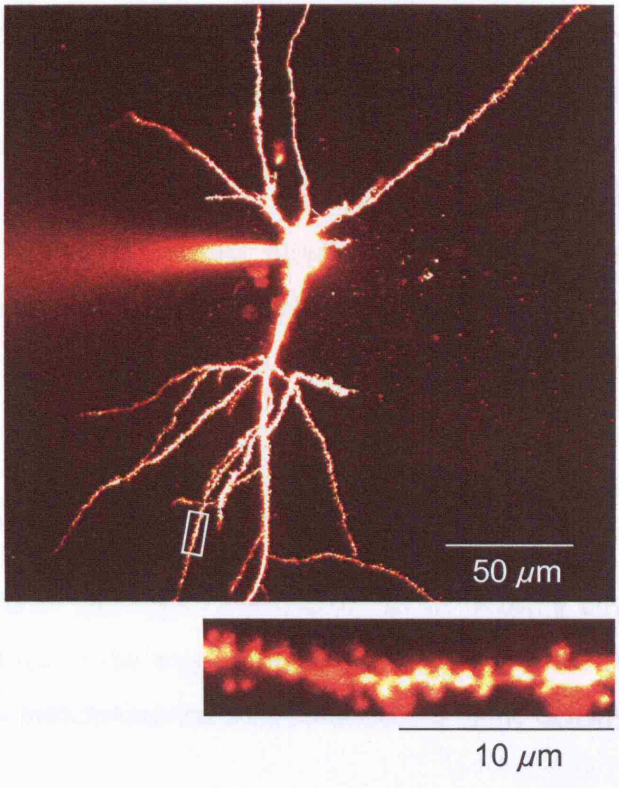
B) Enriched Housing Wild-Type



C) Standard Housing Mutant



D) Enriched Housing Mutant



3.4.3.1 Apical dendrites

Enrichment caused a highly significant decrease in spine density (P = 0.0029) in the apical dendrites of CA1 pyramidal neurones (figure 3.15 A) – an effect that was independent of genotype (P = 0.7383). Like the changes in mEPSC amplitude, this was an unexpected result as spine densities in various brain brains, including the hippocampus, have been reported to increase with environmental enrichment (Moser et al., 1994b;Rampon et al., 2000b;Johansson and Belichenko, 2002;Kolb et al., 2003b). Spine densities were 11% lower in enriched wild-types and 19% lower in enriched mutants compared to their standard environment counterparts. However, Bonferroni post-tests show that the effects of enrichment were only statistically significant in the mutant animals ($P < 0.05$). There was no interaction between genotype and housing condition (P = 0.3462).

3.4.3.2 Correlation analyses to investigate the extent of co-variability between dendritic location and spine density

The dendritic location of each section studied was assessed – defined by its radial distance from the cell soma – and plotted against spine density (figure 3.15 B). Although there is a trend for spine density to increase with distance from the soma (P = 0.075; Pearson's correlation statistics), the fraction of covariance is very low (r^2 = 0.0377) which indicates that less than 4% of the variation in spine density can be explained by variation in dendritic location. Furthermore, there are no significant differences between the dendritic locations of sections studied per animal group (effect of genotype, P = 0.335; effect of housing condition, P = 0.4943; interaction, P = 0.4160); the mean distances are shown in table 3.2. Thus the lower spine densities observed in the enriched mouse groups is not related to the selected dendritic sections analysed.

3.4.3.3 Basal dendrites

There was no effect of either genotype (P = 0.1555) or of housing environment (P = 0.3396) upon spine densities in the basal dendrites of CA1 neurones (figure 3.16 A). This result means that the enrichment induced reduction in spine density in the mutant

mice, was specific to the apical dendrites – the target of Schaffer collateral inputs.

With the exception of the mutant mice that were raised in standard housing conditions, there were no significant differences in spine density between apical and basal dendrites (two-way t-tests). In the mutant animals from standard housing environments, spine densities in the basal dendrites were 20% lower than in the apical dendrites; a difference that was statistically very significant ($P = 0.0054$; figure 3.16 B).

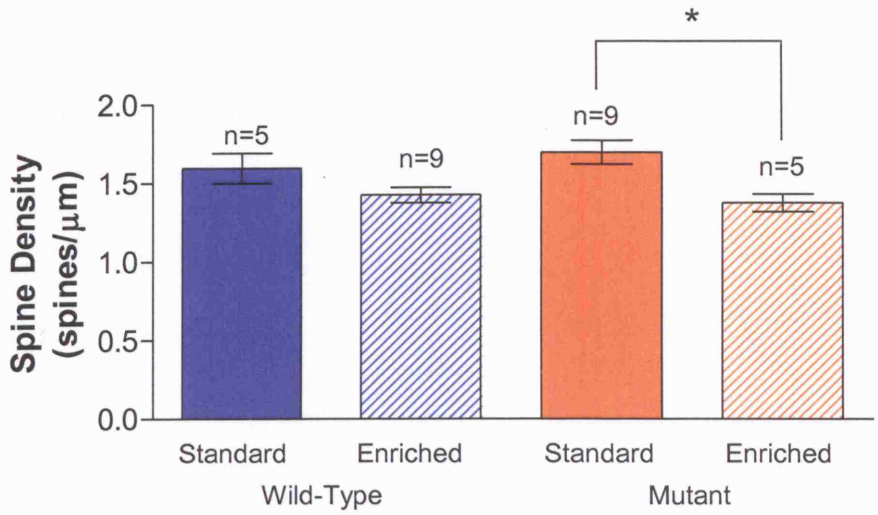
Figure 3.15 Effects of the αCaMKII^{T286A} Mutation and Environmental Enrichment upon Apical Dendrite Spine Density in Adult CA1 Pyramidal Neurones.

Statistical analysis performed using two-way ANOVA and Bonferroni post-tests; * $P<0.05$.

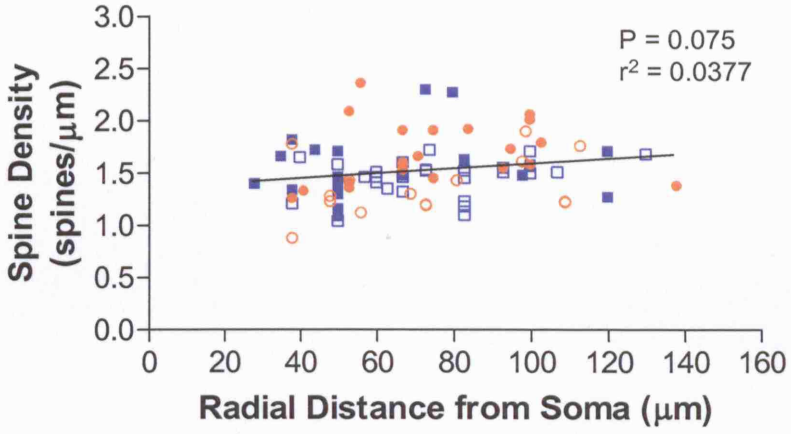
A) Spine densities were assessed using high-resolution confocal images of CA1 neurones. Bar graph shows mean spine densities (± s.e.m.; n cells) within the stratum radiatum region of the hippocampus on the secondary / tertiary branches of the CA1 apical dendrites per animal group. There was no interaction between genotype and housing condition upon spine density ($P = 0.3462$), nor was the effect of genotype significant ($P = 0.7383$); however, the effect of housing condition was very significant ($P = 0.0029$). Bonferroni post-tests show that the effect of enrichment upon spine density was specific to the mutant mice ($P < 0.05$; wild-types, $P > 0.05$).

B) The radial distance of each apical dendrite section studied from the soma is plotted against the section's spine density. The line of best fit and Pearson correlation statistics are shown.

A)



B)



- Standard Housing Wild-Type
- Enriched Housing Wild-Type
- Standard Housing Mutant
- Enriched Housing Mutant

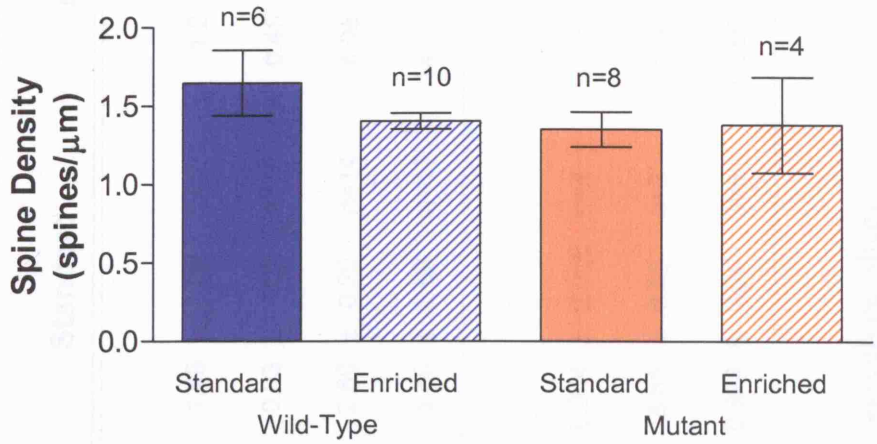
Figure 3.16 CA1 Basal Dendrite Spine Densities are unaffected by the αCaMKII^{T286A} Mutation and Environmental Enrichment.

Bar graphs show means ± s.e.m.; n = number of cells. Statistical analysis performed using two-way ANOVA..

A) Spine densities on CA1 basal dendrites were not effected by genotype (P = 0.1555) or housing condition (P = 0.3396), and there was no interaction between these two factors (P=0.2090).

B) A bar graph showing mean spine densities (± s.e.m.; n cells) for both apical (**A**) and basal (**B**) dendritic regions. In the mutant mice raised in standard housing conditions, spine densities were significantly lower in the basal dendrites than in the apical dendrites (P = 0.0054; two-way t-tests). For the other animal groups, mean spine densities between apical and basal dendrites did not significantly differ.

A) CA1 Basal Dendrites Spine Densities



B) CA1 Apical and Basal Dendrite Spine Densities

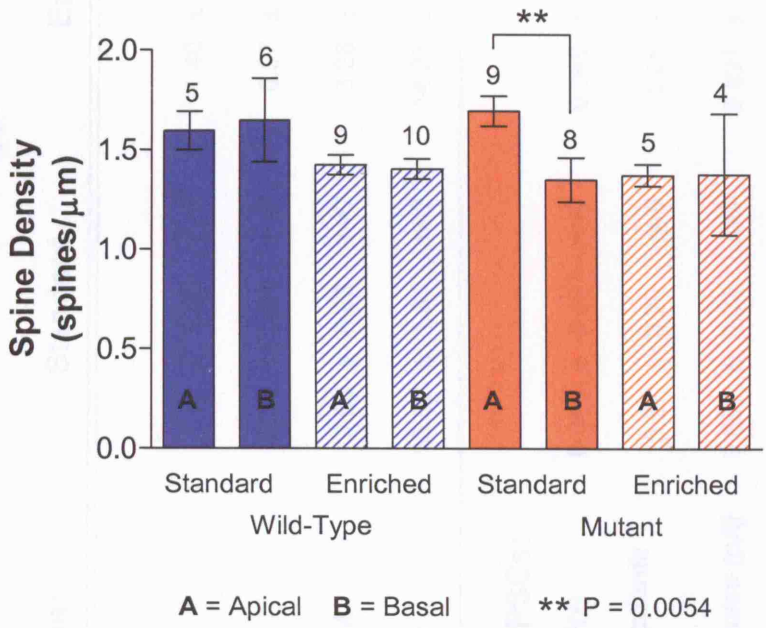


Table 3.1 Summary of Electrophysiology Data.

	Wild-Type Mice				Mutant Mice			
Housing condition:	**Standard**		**Enriched**		**Standard**		**Enriched**	
Evoked EPSCs:								
PPR	1.37 ± 0.06	n=7	1.40 ± 0.05	n=21	1.49 ± 0.08	n=17	1.2 ± 0.03	n=10
Pr failures	0.59 ± 0.04	n=5	0.57 ± 0.03	n=14	0.63 ± 0.02	n=11	0.45 ± 0.04	n=10
EPSC1 amplitude (pA)	3.60 ± 0.63	n=7	3.28 ± 0.30	n=20	2.60 ± 0.20	n=14	4.08 ± 0.36	n=8
EPSC1 decay τ (ms)	15.50 ± 1.81	n=7	14.87 ± 0.78	n=19	15.80 ± 0.68	n=17	14.50 ± 1.77	n=9
Spontaneous mEPSCs:								
mEPSC frequency (Hz)	0.040 ± 0.007	n=9	0.040 ± 0.007	n=6	0.044 ± 0.008	n=8	0.029 ± 0.005	n=9
mEPSC median amplitude (pA)	5.57 ± 0.20	n=9	5.05 ± 0.04	n=6	5.85 ± 0.20	n=8	5.06 ± 0.15	n=9
Mean S.D. baseline noise (pA)	0.890 ± 0.03	n=9	0.897 ± 0.02	n=6	0.924 ± 0.04	n=8	0.880 ± 0.06	n=9

N.B., ≥3 animals were used per animal group in all experiments.
Values presented indicate: the mean ± s.e.m; n = number of cells (no more than one cell per slice).

Table 3.2 Summary of CA1 Neurone Anatomical Measurements.

	Wild-Type Mice		Mutant Mice	
Housing condition:	**Standard**	**Enriched**	**Standard**	**Enriched**
Apical dendrites:				
Mean spine density; n=cells (n of mice)	1.60 ± 0.10 n=5 (3)	1.42 ± 0.05 n=9 (5)	1.69 ± 0.08 n=9 (7)	1.37 ± 0.05 n=5 (3)
Total dendritic length analysed	583 μm	785 μm	727 μm	420 μm
Mean section distance (μm) from soma*; n=sections	65 ± 28.2 n=21	75 ± 21.4 n=26	75 ± 23.5 n=23	74 ± 26.7 n=15
Basal dendrites:				
Mean spine density; n=cells (n of mice)	1.65 ± 0.21 n=6 (3)	1.40 ± 0.05 n=10 (6)	1.35 ± 0.11 n=8 (7)	1.38 ± 0.30 n=4 (3)
Total dendritic length analysed	309 μm	741 μm	712 μm	236 μm

Spine density = spines / μm
Values presented indicate: the mean ± s.e.m.
* Range of distances = 28 – 138 μm

CHAPTER 4
Discussion

4.1 Overview

As discussed in the introduction of this thesis, it is well documented that long-lasting changes in synaptic strength can occur in response to certain patterns of neuronal activity and numerous lines of evidence as well as theoretical studies support the proposal that these changes contribute to the brain modifications that underlie learning and memory (Martin et al., 2000;Martin and Morris, 2002). LTP and LTD, the respective activity-dependent strengthening and weakening of synapses, are currently the best cellular models of synaptic plasticity and can be induced experimentally. However, it is the mechanisms that underlie the persistent strengthening of synaptic connections in the hippocampus that have received the most experimental investigation. Indeed, the wealth of data correlating impairments in the ability to induce LTP with disrupted learning and memory abilities (brought about, for instance, by pharmacological or genetic manipulation, or occurring by natural variation between inbred mouse strains; e.g. Morris et al., 1986;McNaughton et al., 1986;Castro et al., 1989;Giese et al., 1998;Rotenberg et al., 2000;Nguyen et al., 2000;Jones et al., 2001a;Jones et al., 2001b;Bejar et al., 2002) has made a strong case for the proposal that LTP-like mechanisms also occur *in vivo*. At the very least, these studies have provided strong evidence that the identified molecules that are essential for experimentally-induced plasticity, like CaMKII, are also required for learning and memory in animals.

It has been proposed that a major function of the hippocampus in rodents is related to the processing and learning of spatial information (O'Keefe, 1999). If the plasticity of synaptic strength is crucial for this function then it might be considered surprising that measures of baseline synaptic efficacy and connectivity in the hippocampus are often reported to be indistinguishable between wild-type mice and transgenics which show impaired, or even lack, LTP (e.g. Silva et al., 1992b;Abeliovich et al., 1993;Giese et al., 1998;Takahashi et al., 1999;Wong et al., 1999;Ho et al., 2000;Huang et al., 2000;Jones

et al., 2001a;Komiyama et al., 2002;Lee et al., 2003b). This incongruity has also been found in other brain structures where synaptic plasticity is similarly considered an important process underlying their respective functions (e.g. Storm et al., 1998;D'Alcantara et al., 2001). Importantly these reported LTP-null transgenics do not show measurable defects in synaptic transmission, only in the ability to express experimental LTP.

It is certainly possible that either the differences are not being detected (due to a variety of possible reasons that will be discussed below) or that the cellular processes that underlie hippocampal-dependent memory do not result in changes in synaptic strength. I have addressed the first possibility by studying in more detail the properties of excitatory synapses in single hippocampal CA1 neurones from αCaMKII^{T286A} mutant mice compared to their wild-type littermates. Using whole-cell voltage-clamp techniques, I have investigated excitatory synaptic transmission in single CA1 neurones by recording both minimally evoked synaptic responses and miniature EPSCs. In addition, I have compared spine densities between wild-type and mutant CA1 neurones to assess possible differences in the density of excitatory synapses.

As well as studying the properties of excitatory synapses in mice raised in standard lab environments, I have also studied animals that were raised in enriched environments. The enriched environment was used in order to increase the opportunity for the mice to form hippocampal-dependent memories and thus promote physiological (i.e. behaviour-induced) brain plasticity. The hypothesis was that if hippocampal-dependent learning and memory require the modification of synaptic strengths or numbers in the hippocampus, enrichment would increase any differences in synaptic properties between the wild-type and αCaMKII^{T286A} mutant animals and, in turn, increase the chances of detecting such differences. Importantly, environmental enrichment does not rescue the spatial learning deficits found in αCaMKII^{T286A} mutant mice (Need and Giese, 2003); therefore, alternative processes are unable to compensate for the cognitive deficit in these mutant animals.

In summary, I found that when animals were raised in the standard housing conditions, the loss of Thr286 autophosphorylation in the mutant animals had no significant effect

upon the basal measures of Schaffer collateral synaptic efficacy. This stands in agreement with the findings of previous transgenic studies (see above) that also claimed basal levels of synaptic transmission were unaltered in LTP-null mice. One unexpected finding, however, was that the rise-times of mEPSCs recorded from CA1 neurones were significantly faster in the mutant mice compared to the wild-types. Some possible explanations for this finding will be discussed.

Enrichment did result in significant changes in the basal synaptic properties of CA1 neurones. However, contrary to the hypothesised outcome, the significant changes to mean values were only found in the mutant animals, whilst the mean values of the measured properties of wild-type neurones remained stable. The only significant change noted for the wild-type data was that the distribution of median mEPSC amplitudes across cells (but not the mean value) was altered such that median mEPSC amplitudes from enriched mice became more similar to each other. The initial hypothesis that the effects of enrichment would be primarily observed in the wild-type animals and blocked in mutants was therefore incorrect. In the mutant mice, enrichment caused an increase in the averaged amplitude of evoked unitary EPSCs. This was most likely due to an underlying increase in presynaptic release probability – as indicated by lower failures rates and higher levels of paired-pulse facilitation. In addition, the median mEPSC amplitude was reduced in the mutants although the frequency of events remained unchanged. In the sections below, I present some possible interpretations of these unexpected findings.

The remainder of this Ph.D. work was dedicated to providing information on the expression of wild-type αCaMKII during the first postnatal week of development and investigating whether the manipulation of the αCaMKII gene may have interfered with the expression of CaMKII in the mouse brain. These studies are discussed first.

4.2 αCaMKII Expression and Distribution in Wild-type and Mutant Mouse Brain through Post-Natal Development

It has previously been shown that levels of αCaMKII protein in the hippocampus (Hardingham et al., 2003) and in total brain (Giese et al., 1998) are unchanged in adult

αCaMKII^{T286A} homozygous mutant mice compared to wild-type littermates. It has also been shown that levels of βCaMKII are normal in the mutants (Giese et al., 1998). Together these findings suggest that expression of the αCaMKII gene is unaltered by genetic manipulation in this transgenic mouse and that the lack of αCaMKII Thr286 autophosphorylation does not result in compensatory up-regulation of either subunit in the hippocampus.

Using *in situ* hybridization techniques, I have additionally shown that the mutation neither affects the spatial nor temporal control of expression within the first few days of postnatal development. For instance, the observed developmental increases in CaMKII expression within the CA1 sub-region of the hippocampus, the dentate gyrus and the cortex of the wild-type brain are accurately mirrored in the mutant brain. These findings consolidate the conclusion that neither the T286A point-mutation nor the residual loxP site (leftover from the Pointlox gene-targeting strategy used by Giese et al 1998) alters αCaMKII gene expression. If CaMKII gene expression is unaltered through development in the mutant mice, it is likely that the Ca^{2+}/CaM-dependent functions of CaMKII are also unaltered through development. This assumption is supported by the fact that levels of Ca^{2+}/CaM-dependent CaMKII activity are unaltered in adult hippocampal tissue (Giese et al., 1998). Thus, any mutant phenotype most likely results from the specific lack of Ca^{2+}/CaM-independent kinase activity and not due to altered kinase expression though development.

It must be noted, however, that significantly reduced levels of αCaMKII protein have been found in both the somatosensory cortex (Hardingham et al., 2003) and the visual cortex (Taha et al., 2002) of adult αCaMKII^{T286A} mutant mice compared to wild-types. Thus, regional differences in either the expression or translation of cortical mRNA are known to occur in the mutant mouse. The reduced levels in the mutants may be a direct consequence of their impaired synaptic plasticity, caused by the loss of experience/plasticity-dependent increases in expression that remain intact in the wild-type brain and result in the differences found. Also note, that although it has been hypothesised that increases in CaMKII concentration (at least within the potentiated synapses) could be important for the maintenance of LTP (Chen et al., 2001;Lisman and Zhabotinsky, 2001), it is still not clear whether persistent increases in CaMKII

expression are necessary. Interestingly, in the study of Hardingham et al (2003), protein levels in the hippocampi of the same animals were found to be unaffected, despite the fact that hippocampal LTP is also severely impaired (Giese et al., 1998). Perhaps a reason for this disparity is due to the differential functions of the principal cells in the cortex versus those in the hippocampus regarding the long-term plasticity/storage of memory. If permanent memory traces are held within circuits of potentiated synapses in the cortex and if this does involve an increase in the synaptic concentration of CaMKII (or synapse density), then a continual and persistent accumulation of CaMKII in the cortical tissue might occur as the archives of memory are continually expanded. On the other hand, if potentiated synapses in the hippocampus are subsequently de-potentiated or depressed as memories are transferred out to the cortex for final storage, net levels of the kinase may not change over time. Alternatively, the cognitive functions of the hippocampus may simply be less readily recruited during the life of a standard laboratory-reared animal compared to greater levels of synaptic processing occurring in the cortex; this might account for the lack of difference in hippocampal CaMKII expression levels between the genotypes.

Although I have not investigated levels of βCaMKII expression in the mutant mice throughout development (note, no differences were found at adulthood compared to wild-types; Giese et al., 1998), there are several reasons why one might not expect changes to occur. Firstly, a recent study has shown that a 3-fold over-expression of αCaMKII in hippocampal neurones had no effect upon the detected levels of βCaMKII in the soma of the same cells, relative to untransfected neruones (Thiagarajan et al., 2002). Interestingly, this was in contrast to an observed 44% decrease in the levels of αCaMKII when levels of βCaMKII were similarly increased (Thiagarajan et al., 2002). Thiagarajan et al also demonstrated that increased *amounts* of αCaMKII protein (following transfection) resulted in synaptic changes that reflected the altered levels of αCaMKII *activity*; they observed increased mEPSC amplitudes and a reduction in mEPSC decay time. Such observations agree with the known effects of CaMKII in promoting the synaptic insertion of the more rapidly decaying GluR1-containing AMPA receptors (Hayashi et al., 2000); i.e. compared to the slower kinetics of Glu2/GluR3 containing AMPA receptors. Thus, changes in αCaMKII *protein* and *activity* levels did not cause compensatory alterations in βCaMKII protein expression. Indeed, even when

mice have a complete lack of αCaMKII, the total levels of βCaMKII in adult mice are still found to be unaltered in both whole forebrain (Silva et al., 1992b) and hippocampus-only homogenates (Elgersma et al., 2002). In this study, the specific loss of Thr286 autophosphorylation-dependent αCaMKII activities and functions through development would not, therefore, be expected to cause changes in the levels of βCaMKII.

Secondly, in contrast to the α-subunit, it is known that expression levels of βCaMKII in the rodent brain are already high during early development, possibly implicating a more dominant role for this isoform in processes involved in brain development. βCaMKII is already prominent in the brain at embryonic day 16.5 (Bayer et al., 1999); in fact, total brain levels decrease slightly during the first weeks of postnatal life to reach stable adult brain intensities by 3 weeks of age (Burgin et al., 1990). This decrease in expression is specific to the cortex; in the hippocampus, levels were found to be at adult intensities at P4 and to remain stable until at least 3.7 months of age (Burgin et al., 1990). This is in contrast to the α-subunit, where respective 10- and 5-fold increases in mRNA levels are seen to occur in the cortex and hippocampus during postnatal development; the majority of these increments occurring during the second postnatal week (Kelly and Vernon, 1985;Kelly et al., 1987;Burgin et al., 1990). It does not seem probable therefore that the loss of αCaMKII Thr286 autophosphorylation would result in compensatory up-regulation of the already more prevalent βCaMKII subunit.

In summary, it is unlikely that any differences, or lack of differences, found between the hippocampal properties of the wild-type and mutant animals are consequent to compensatory changes occurring in the levels of either the α or β CaMKII subunits. Comparable levels of the αCaMKII between the genotypes, at the ages studied, also suggests that the Ca^{2+}/CaM-dependent functions of the enzyme would be unaltered in the mutant mouse through development. Thus, any physiological differences found in the adult mutant animals most likely result from the lack of αCaMKII-dependent processes that require the autophosphorylation of Thr286.

4.2.1 CaMKII is Prevalent in the Hippocampus from Birth

A previous study of the developmental expression profile of the mouse CaMKII subunit mRNAs (using Northern blot analysis in whole brain) was unable to detect the presence of αCaMKII until P5 (Bayer et al., 1999). This was in contrast to the profile of the other subunits, where they were able to detect signals for β, γ and δ CaMKII mRNAs at various embryonic stages. Using the more sensitive *in situ* hybridization technique in parasagittal brain slices they were, however, able to detect a signal for the α subunit in P1 hippocampus that was restricted to the CA2 sub-region. Thus, Bayer et al could only conclude that αCaMKII was expressed from birth but not in all regions that show expression at more mature ages. No other study of αCaMKII expression through development has looked in brain before postnatal day 4.

This study of αCaMKII expression is consistent with the findings of Bayer et al (1999), in that the highest levels of mRNA, and protein, are found in CA2; however, we also detect an equally strong signal within CA3, plus lower levels in CA1 and the inner blade of the dentate gyrus. Significant levels of αCaMKII mRNA are also found in the entorhinal cortex, the striatum and the olfactory bulb of P1 mouse brain. The more pronounced and wider distribution of staining seen here therefore challenges the supposition that αCaMKII is only expressed postnatally. Investigations using these same techniques in embryonic brain is required to understand when the true onset of αCaMKII expression occurs – and also, therefore, the potential onset of CaMKII-dependent processes.

4.2.2 CaMKII & Brain Development

Maximal levels of αCaMKII are reported to be reached during the second to third postnatal weeks of development (Kelly and Vernon, 1985;Kelly et al., 1987;Burgin et al., 1990) and this has been noted to correlate with both the most active period of synaptogenesis in the hippocampus (Burgin et al., 1990) and with the onset of 'adult-like' CaMKII-dependent forms of synaptic plasticity (Yasuda et al., 2003a). However, the strong and wide distribution of expression of αCaMKII at the younger ages, as seen here, suggest that αCaMKII is also likely to be involved with neuronal function in the developing brain.

Although the formation of structural synaptic contacts in the brain is thought to occur in the absence of neurotransmitter secretion (Verhage et al., 2000; but see Ben Ari, 2001) and can even be enhanced by NMDA receptor blockade (Lüthi et al., 2001), correlated neural activity and plasticity of synaptic strengths is thought to play an important role in the appropriate refinement of connections and the establishment of functional networks in the brain (Goodman and Shatz, 1993;Katz and Shatz, 1996;Constantine-Paton and Cline, 1998;Huh et al., 2000). It had also been proposed that the mechanisms underlying the strengthening of selected inputs during development may recruit mechanisms of potentiation that are akin to those underlying LTP in the adult brain (Durand et al., 1996;Liao and Malinow, 1996;Isaac et al., 1997;reviewed in: Constantine-Paton and Cline, 1998;Feldman et al., 1999). However, the lack of any gross defects in brain morphology or simple animal behaviours in the αCaMKII-null and T286A transgenic mice (Silva et al., 1992b;Giese et al., 1998) as well as no apparent compensatory up-regulation of the α subunit, as shown here, do imply that fully intact αCaMKII functioning is not critical for general brain development and that the developmental forms of plasticity involved in the formation of functional networks occur via αCaMKII-independent pathways in the transgenic mice.

Indeed, in the hippocampus, CaMKII *independent* forms of experimental synaptic potentiation have been demonstrated within the first two weeks of postnatal life (Wikström et al., 2003;Yasuda et al., 2003a). One mechanism that could be recruited for the strengthening of contacts during the first post-natal week of development might involve the synaptic insertion of GluR4 subunit-containing AMPA receptors; a process shown to be independent of CaMKII activity (Zhu et al., 2000). Importantly, such observations of alternative plasticity mechanisms in developmentally immature brain tissue do not imply that αCaMKII-dependent forms of plasticity do not also exist (e.g. Wu et al., 1996), but it suggests that the plasticity of connections may also occur via parallel signaling pathways; for example, via PKA or PKC activity (Wikström et al., 2003;Yasuda et al., 2003a). Another candidate molecule is also of course the βCaMKII subunit which, as discussed above, is already prominent by embryonic day 16.5 (Bayer et al., 1999). It would be interesting to see if developmental abnormalities occur in a transgenic mouse where the autophosphorylation of both α and β subunits is blocked.

In further support of age-specific mechanisms of synaptic plasticity, an age-dependent decrease in synaptic plasticity was observed in the neocortex of αCaMKII-knockout mice (Kirkwood et al., 1997). This study was able to illicit wild-type levels of LTP in homozygous mutant animals of 4-5 weeks of age, but not in adults (> 6 months old) – implicating αCaMKII-independent mechanisms to be more prevalent in the younger animals. Caution is required, however, in interpreting this observation due to the use of the αCaMKII-null mutant since intact experience-dependent plasticity in the somatosensory cortex of 4-8 week old knock-out animals (Glazewski et al., 1996) was subsequently found to be severely impaired in $CaMKII^{T286A}$ transgenic animals of the same age (Glazewski et al., 2000).

It has been proposed that the residual hippocampal LTP observed in adult αCaMKII-null mice may be due to increased translocation of βCaMKII to synaptic sites (Elgersma et al., 2002). The age-dependence decrease in cortical plasticity seen in the experiments of Kirkwood et al. (1997) could also be explained therefore by a higher capacity for the younger knockout animals to compensate through greater levels of compensatory βCaMKII translocation (Elgersma et al., 2002). No such compensation through βCaMKII translocation was found in the T286A mutants (Elgersma et al., 2002).

These points highlight the elegance of using a point mutation to generate only a partial loss of function to block CaMKII-dependent potentiation since compensation by the formation and action of βCaMKII homomeric enzymes is not brought about and the mutant α-subunits (as far as it is known) remain an integral part of the CaMKII holoenzyme.

4.3 Effects from the loss of Thr286 Autophosphorylation in CA1 Pyramidal Neurones of Mice Raised in Standard Housing

If CaMKII-dependent modification of synaptic strength and number is occurring in the functioning hippocampus where the plasticity of synapses is thought to be key to its role in memory formation, it might be expected that experience and plasticity would result in electrophysiological and/or morphological differences between the genotypes. Synaptic

potentiation induced *in vitro*, has been shown to correlate with changes in the number of functional synapses (e.g. Durand et al., 1996;Poncer and Malinow, 2001;Reid et al., 2004), synapse and spine number/density (Chang and Greenough, 1984;Chang et al., 1991;Dempster, 1993;Maletic-Savatic et al., 1999;Engert and Bonhoeffer, 1999;Toni et al., 1999;Jourdain et al., 2003;Nägerl et al., 2004) and in the properties of short-term plasticity (e.g. Kleschevnikov et al., 1997;Sokolov et al., 1998). Thus, in addition to assessing baseline measures of synaptic efficacy at Schaffer collateral inputs in wild-type and αCaMKII^{T286A} mutant mice using minimal stimulation, recordings of mEPSCs were made as a measure of the total excitatory input to CA1 neurones, and spine densities were calculated to assess the density of axospinous synapses within select dendritic regions and potentially to reveal possible structural effects that may result from the loss of CaMKII autophosphorylation.

In the first study of the CaMKII^{T286A} mutant mice, Giese et al. (1998) reported that the gross levels of synaptic connectivity between Schaffer collateral axons and CA1 pyramidal cells were unaltered in the mutant hippocampus compared to wild-type litter-mates. They showed that the relationship between the amplitude of evoked fibre-volleys (a measure of pre-synaptic activation) and excitatory field potentials (a measure of postsynaptic depolarisation) was indistinguishable between genotypes. Although these measurements do indicate that the summed responses recorded from a large number of synapses are not altered in the αCaMKII^{T286A} mutant, it gives no information about possible differences at the level of individual synapses. For instance, it may be that differences in synaptic weight within a subset of the inputs are masked by compensatory changes occurring at other synapses in order to maintain the excitability of the neuron (Turrigiano et al., 1998;Turrigiano and Nelson, 2004). Thus, differences in the measured properties at individual synapses might be seen in wild-type neurones compared to the αCaMKII^{T286A} mutant neurones. Alternatively, net increases or decreases in synaptic strength could be equilibrated by changes in synapse number. Neither of these points had previously been addressed in the CaMKII^{T286A} mutant mouse. Thus, the starting point of these investigations was based upon the hypothesis that differences may exist between the wild-type and αCaMKII^{T286A} mutant mice in the properties of excitatory connectivity at the level of individual inputs.

4.3.1 *Basal Measures of Pre- and Post-Synaptic Efficacy are Indistinguishable between Genotypes*

In wild-type and mutant mice raised in standard laboratory housing I found no significant differences in the properties of evoked unitary EPSCs arising from paired-pulse minimal stimulation of Schaffer collateral axons, no differences in the frequency or amplitude of mEPSCs and no significant differences in the density of spines within proximal to middle regions of the apical dendrites or in the basal dendrites.

Although CaMKII-dependent (experimentally-induced) plasticity is known to be blocked in the hippocampus and neocortex of CaMKII^{T286A} mutant mice (Giese et al., 1998;Hardingham et al., 2003), it might be proposed that the lack of differences in these basal measures of synaptic efficacy between genotypes could reflect the existence of alternative mechanisms that can compensate for the loss of CaMKII-dependent plasticity. This is not a plausible explanation, as hippocampal-dependent learning and memory as well as experience-dependent plasticity in both the somatosensory and visual cortices remain impaired in these mutant mice (Giese et al., 1998;Glazewski et al., 2000;Taha et al., 2002;Need and Giese, 2003). Much larger numbers of recordings would be required to confirm whether or not the distributions of synaptic properties are similar between genotypes (see below for further discussion of this point; section 4.3.3).

4.3.2 *Miniature EPSC Rise-Times are faster in CA1 Neurones from the CaMKII^{T286A} Mutant Mice*

An unanticipated difference between the genotypes was the observation that mean mEPSC rise-times were significantly faster in the mutant mice (irrespective of housing condition). There are many factors that contribute to the kinetics of somatically detected EPSCs that could be altered in the mutant CA1 neurones. For instance, distinct properties of the mutant CA1 neurones may lay within the synapse or in the structure of the neurones that would in turn affect the extent of dendritic filtering.

4.3.2.1 Faster Rise-Times may arise from Distinct Synaptic Properties

The shorter rise-times of mutant mEPSCs could be the result of distinct synaptic properties which reduce the most time-limiting step of synaptic transmission that is

receptor activation following glutamate release and diffusion across the cleft. The underlying factors that determine the kinetics of this step and thus the postsynaptic response include a) the time-course and magnitude of transmitter concentration in the cleft, b) the gating properties of the postsynaptic receptors and c) their subunit composition (Jonas and Spruston, 1994).

With respects to a) the time-course and magnitude of transmitter concentration, differences in quantal content, the manner in which transmitter is released (e.g. due to the dynamics of the fusion-pore opening; Choi et al., 2000), synaptic cleft morphology or the kinetics of transmitter clearance could all affect the rate of postsynaptic receptor activation and therefore the rise-times of mutant mEPSCs. Models of synaptic transmission have also predicted that the spatial distribution of postsynaptic receptors within a synapse can also affect their rate of their activation (e.g. Wahl et al., 1995), although the spatial distribution of receptors in wild-type neurones would have to be substantially larger in order to account for the differences seen here between genotypes.

It is not clear how the lack of CaMKII autophosphorylation and its dependent forms of plasticity could have resulted in differences in any of these factors. Additionally, it is unlikely that differences exist between the genotypes with respects to b) and c). The gating kinetics of AMPA receptors are not known to be affected by either CaMKII or by other molecules involved in synaptic plasticity (in contrast with AMPA receptor conductance; Benke et al., 1998). Although differences between the genotypes in AMPA receptor subunit composition are possible, and even likely due to the probable loss of CaMKII-dependent insertion of GluR1 containing AMPA receptors, it is improbable that this could have contributed towards the observed difference in rise-times because the gating of AMPA receptors by glutamate is extremely fast and does not differ significantly between subunits (Dingledine et al., 1999;Grosskreutz et al., 2003).

If synaptic differences were the source of this difference between wild-type and mutant mEPSC rise-times, then faster rise-times would also be expected for evoked EPSCs in the mutant mice. In this study, rise-times for the evoked EPSCs were not assessed due to difficulty in accurately aligning the events on their rise. The source of the problem was that the levels of baseline noise were generally higher in these recordings. For

the measurements of peak amplitudes and decay time constants, evoked responses were averaged with respect to the stimulus artifact. Thus, it was not as crucial to obtain such low baseline noise levels as it was for detection of mEPSCs which were measured individually. Averaging the traces in this manner has a minimal effect upon amplitude or decay time measurements, but the small amount of delay jitter between stimulus and EPSC onset among stimulation sweeps had a large effect upon rise-time measurements and thus can not be assessed using events averaged in this way.

4.3.2.2 Faster Rise-Times may arise from Distinct Electrical Features of Mutant CA1 Neurones

Excitatory synapses are located throughout the extensive dendritic trees of CA1 neurones and currents measured at the soma, in particular those that arise at more distal locations, are therefore highly filtered with respects to both time course and amplitude (Stuart and Spruston, 1998;Jaffe and Carnevale, 1999;Magee and Cook, 2000;Williams and Stuart, 2002). The extent that synaptic currents are filtered is dependent upon both the passive and active properties of neuronal membranes. Differences in active conductances would have been minimized as a result of whole-cell voltage-clamp, but as the clamp is far from perfect, particularly in the more distal regions of the dendritic tree, active conductances may still be in operation; thus such differences between the genotypes cannot be excluded. Passive filtering, on the other hand, is primarily governed by the combination of resting 'leak' conductances (Stuart and Spruston, 1998) and features of the neuronal architecture; in particular, the geometry of the primary apical dendrite (Jaffe and Carnevale, 1999). Leak conductances would also have been minimised in these recordings due to the use of a CsCl-based internal solution. Cs^+ ions block open K^+ channels in the membrane which, under these recording conditions, would constitute the majority of any leak conductance.

Clues about the electrical properties of the CA1 proximal apical dendrites could have been gained from rise-time analysis of Schaffer collateral evoked EPSCs which are likely to arise from comparable positions within the proximal region of the apical dendrites. However, for the reasons discussed above, evoked EPSC rise-times were not assessed in these experiments. Nevertheless, it has been shown in αCaMKII knockout mice that the time-courses of both AMPA and NMDA receptor mediated synaptic

currents evoked within *stratum radiatum* are indistinguishable from wild-types (Silva et al., 1992b). This suggests that differences in dendritic filtering due to altered electronic properties on the proximal dendrites are unlikely to exist in the knockout animals and it is likely that the same be true for the T286A transgenics.

Differences in the electronic passive filtering properties of dendrites determined by their complement of ion channels is unlikely therefore to be the cause of the faster mEPSCs rise-times in the mutant cells. It should not, however, be ruled out as a possibility, especially as various dendritic K^+ ion channels have been found to be targets of CaMKII phosphorylation, where phosphorylation specifically affects their level expression in the membrane (e.g. Varga et al., 2004;Sergeant et al., 2005).

4.3.2.3 Faster Rise-Times may arise from Distinct Morphological Features of Mutant CA1 Neurones

Alternatively, the faster average rise-times in the mutant animals could result from a larger proportion of events being subject to less dendritic filtering due to differences in dendritic architecture between the genotypes. This could arise if the mutant pyramidal cells have overall electrically shorter dendritic trees, or, if cells are similar in size, this could also arise if a smaller proportion of mEPSCs arise at synapses in the more distal regions of the tree. This could reflect a proximally biased distribution of synaptic densities or a non-uniform distribution of pre-synaptic release probabilities, with those synapses more distal less likely to undergo the spontaneous release of neurotransmitter.

Importantly, mEPSC amplitudes are also attenuated by dendritic filtering, but there is no difference in mean mEPSC amplitude between the wild-type and mutant mice raised in standard housing conditions. *If* the cause of the faster mEPSC rise-times in the mutant mice is that the spontaneous events are occurring on average at electrically more proximal positions within the dendritic tree, then the current transfer at the synapse, i.e. the dendritic amplitude, may also be less in mutant cells in order to result in comparable amplitudes recorded at the soma to those in wild-types.

Smaller synaptic amplitudes would be compatible with a loss of CaMKII function since CaMKII activity is known to increase AMPA receptor conductance following

phosphorylation of the GluR1 subunit at residue Ser831 (Mammen et al., 1997;Barria et al., 1997a) and is required for the activity-dependent insertion of AMPA receptors into synapses (Shi et al., 1999;Hayashi et al., 2000;Andrasfalvy and Magee, 2004).

4.3.2.4 CA1 Dendritic Arborisation may be modulated by CaMKII Activity during Development

How might CA1 morphological differences, such as smaller dendritic arbours, arise from the lack of αCaMKII-dependent processes in the mutant mice? One possibility could lie in the role of CaMKII in developing hippocampal neurones. The ability of the βCaMKII subunit to bind the holoenzyme to the actin cytoskeleton has been shown to play a critical role in promoting dendritic arborisation in neonatal neurones *in vitro* (Fink et al., 2003). If the mechanism by which the actin-associated CaMKII holoenzymes affects neuronal structure requires autonomous activity of the holoenzyme subunits, then such morphogenic effects of CaMKII would indeed be impaired in these $\alpha CaMKII^{T286A}$ transgenic mice.

As miniature EPSCs arising at synapses in the more distal regions of the pyramidal cell dendrites are affected most by the effects of dendritic filtering, it is likely that the faster average mEPSC rise-times seen in the mutants are due to fewer detected events arising in the more distal regions. A reduced extent of distal dendrite arborisation and thus synapse number could account for fewer mEPSCs arising in such regions. Confocal images taken for this thesis were only focused at assessing spine densities within the proximal to mid regions of wild-type and mutant CA1 neurones – the target for the majority of Schaffer collateral axons (Ishizuka et al., 1990;Li et al., 1994). Thus, the distal third of CA1 dendritic arbours were not visualized at all; the largest confocal images taken are likely to capture at most half of the dendritic structure (see section 2.7; figure 2.4). Therefore, identification of possible differences between the two genotypes in the extent of distal dendrite arborisation was not possible.

4.3.3 Could Differences in Synaptic Efficacy Still Exist but Lie Undetected?

If only a small proportion of wild-type synapses exhibit experience-dependent altered

properties at any one time, it is possible that any pre- or post-synaptic phenotypes in the mutants resulting from the loss of CaMKII-dependent plasticity may simply lie undetected rather than not necessarily existing. For instance, it is known that considerable heterogeneity in pre- and post-synaptic properties exists at CA1 synapses (Rosenmund et al., 1993;Hessler et al., 1993;Dobrunz and Stevens, 1997;Murthy et al., 1997;Huang and Stevens, 1997;Hanse and Gustafsson, 2001). For example, Murthy et al. (1997) used FM1-43 dye labelling and de-staining of synaptic vesicles in order to visualize the release probabilities at a large number of synapses in cultured neurones. They found that the distribution of CA1 presynaptic release probabilities was broad and continuous with a predominance of synapses with low release probabilities and a positive skew towards higher values. If such a distribution exists for synapses in hippocampal slices and is shaped by prior synaptic plasticity, in particular the skew to higher probabilities, a significantly different distribution might be expected in the mutant mice. However, thousands of inputs synapse with each CA1 neurone within the stratum radiatum (Megías et al., 2001), thus a very large number of individual recordings would be required in order to be able to detect such a difference in the distribution of release probabilities between genotypes. Assessing how such distributions might be shaped by experience-dependent plasticity through the use of the techniques as used by Murthy et al. (1997) would not be possible for adult wild-type and mutant mice as this method requires the use of cultured neurones obtained from neonatal animals.

If a larger proportion of CA1 synapses in the wild-type mice did boast higher release probabilities compared to the mutants then it might be more feasible to detect such a difference by assessing the frequency of spontaneously occurring mEPSCs which do present information about a much larger proportion of a cell's excitatory inputs. As stated above, no significant differences in the mean frequencies (or amplitude) of miniature events were found between genotypes. This again suggests that overall mean release probabilities are indeed unaltered in the mutants – although this interpretation does assume that the total numbers of excitatory synapses are comparable between genotypes. Although experiments were not performed in order to estimate and thus compare the total number synapses in wild-type and mutant CA1 neurones, there were, at least, no significant effects of genotype upon spine densities in either the proximal to

mid apical or basal dendritic compartments assessed. This does suggest that the densities of axospinous excitatory synapses in these compartments may be similar as it has been previously shown that virtually all spines within the CA1 region of hippocampus form a synapse with a single axon terminal (Harris and Stevens, 1989;Schikorski and Stevens, 1997); although this does not necessarily imply that all spines form functional connections.

It must also be noted that various aspects of transmission are known to be temperature sensitive, including: the frequency of spontaneous events, the time-course and amplitude of synaptic currents, quantal content and the magnitude of paired-pulse facilitation (Barrett et al., 1978;Nikol'skii and Voronin, 1986;Wahl et al., 1995;Volgushev et al., 2004). Indeed, recordings from CA1 neurones in acute hippocampal slices obtained at more physiological temperatures have been reported to yield larger amplitude mEPSCs at frequencies several fold higher than those presented here that were collected at room temperature (e.g. Ghamari-Langroudi and Glavinovic, 1998;Lee et al., 2003b); note, reports also exist where mEPSC frequencies recorded room temperature are substantially greater than those seen in this thesis (e.g. Hsia et al., 1998). The reduced amplitudes of events recorded at room temperature will also have resulted in a larger proportion of events being lost into the baseline noise, leaving only the larger events detectable. Combined with the fact that fewer events are detected within a set time due to the reduction in mEPSC release probabilities, this would have further reduced the opportunity to detect differences (both pre- and post-synaptic in origin) between the genotypes.

4.3.4 Could Synaptic Differences between Genotypes be Reversed Following the Preparation of Hippocampal Slices?

It is also possible that differences in the measures between genotypes do exist *in vivo* but that plastic changes to synapse efficacy or spine number brought about by decapitation, brain slicing and slice maintenance may thereafter mask the *in vivo* phenotype. Indeed numerous structural and biochemical changes in pyramidal neurones have been found to be triggered by hippocampal slice preparation (e.g. Whittingham et al., 1984;Suzuki et al., 1994;Zhou et al., 1995;Siklos et al., 1997;Kirov et al., 1999;Lengyel et al., 2001a;Taubenfeld et al., 2002;Fiala et al., 2003;Ho et al., 2004).

Thus, it can not be ruled that any differences in synaptic properties between the genotypes may have been camouflaged by slicing-mediated changes.

One of the immediate effects of slicing is the complete disassembly of dendritic microtubules; they reassemble back into normal axial arrays within 5 minutes post-slicing but require up to 3 hours to reform microtubule lengths comparable to those seen in perfusion-fixed hippocampi (which would presumably represent the natural *in vivo* state; Fiala et al., 2003). Other immediate but transient effects include metabolic changes such as the depletion of glycogen stores and drops in creatine and ATP levels, (Whittingham et al., 1984;Feig and Lipton, 1990;Fiala et al., 2003).

Other consequences of slicing have been found to develop with time. Large increases in synapse and spine number have been observed in the hippocampus (Wenzel et al., 1994;Kirov et al., 1999), although no changes were reported for the first 3 hours after slicing (Fiala et al., 2003). Substantial changes have also been reported in the protein levels of the GluR1 and GluR3 AMPA receptor subunits (Taubenfeld et al., 2002). GluR1 levels dropped to 46% of control levels within 1 hour after slicing and decreased further still hour on hour down to 15% by 6 hours *in vitro*. GluR3 levels were found to follow a similar pattern, dropping down to 31% of control levels after 6 hours. The control levels for this study were set to those found in hippocampi that were rapidly frozen following removal from the brain. Despite the reduced levels of GluR1, it is interesting that potentiation of field potentials could still be induced in these slices after 6 hours *in vitro*, implying that the levels of GluR1 protein were still sufficient to undergo rapid delivery into the stimulated synapses. This is supported by the observation that LTP in the GluR1 knockout mouse could be rescued by the controlled expression of GluR1 by as little as 10% of normal wild-type amounts (Mack et al., 2001).

Profound de-phosphorylation of the GluR1 AMPA receptor subunit at both the PKA and the CaMKII sites (Ser845 and Ser831 respectively) has also been found following the preparation of hippocampal slices (Ho et al., 2004). This indicates that a shift occurs in the balance between protein phosphatases and protein kinases that favours the net de-phosphorylation of substrates. The de-phosphorylation of both GluR1 sites was rapid (significant after only 30 minutes after slicing), with phosphorylation levels

decreasing to as little as 25% of those seen in unsliced control tissue which showed no signs of recovery after 8 hours *in vitro*. This study found no changes in total GluR1 protein levels compared to unsliced control tissue, contradicting the dramatic changes seen by Taubenfeld et al (2002). Slicing-induced changes to αCaMKII have also been assessed. Ho et al (2004) found that slicing had no effect upon the total levels of αCaMKII protein but did cause rapid and large changes to the levels of Thr286 autophosphorylation and interestingly the direction of change was dependent upon slice maintenance conditions. When slices recovered in interface chambers, Thr286 phosphorylation levels were seen to decrease by 50% within 30 minutes and showed no recovery after 3 hours *in vitro*. In contrast, slices that recovered in submerged chambers (as were used in this thesis) showed 100% higher levels of Thr286 phosphorylation which returned back to control levels after 3 hours *in vitro*. Despite this apparent increase in CaMKII activation, a net 50% de-phosphorylation of GluR1 ensued in slices from the submerged chambers indicating that the balance remained in favour of the phosphatases (Ho et al., 2004). Lengyel and colleagues (2001) also found that hippocampal slice preparation lead to vastly elevated levels of Thr286 autophosphorylation. They also demonstrated that levels of CaMKII autonomous activity were increased, although this rise subsequently decreased by 70% after 2 hours of recovery. This study found that decapitation was the stimulus for CaMKII activation, lieing in agreement with a previous study that found decapitation leads to a marked translocation of CaMKII to the PSD (Suzuki et al., 1994) – a redistribution of the kinase that requires the binding of Ca^{2+}/CaM to disrupt its association with the actin cytoskeleton within neuronal processes (Shen and Meyer, 1999). The control levels of CaMKII autophosphorylation in freshly cut slices used by Ho et al. (2004) may therefore represent an overestimation of the baseline levels *in vivo*.

Together, these results suggest that the slicing procedure could potentially lead to either the depotentiation or depression of synaptic responses; the latter arising from the de-phosphorylation of GluR1 at Ser831 (which would reduce AMPA receptor conductances; Derkach et al., 1999) and the de-phosphorylation at Ser845 (which would lead to the removal of GluR1-containing receptors; Kameyama et al., 1998;Lee et al., 2000). These findings also have important implications for studies of the plasticity of synaptic efficacy in the hippocampus which would be expected to be highly altered

compared to the properties of synapses *in vivo*. The de-phosphorylation of Ser845 might be expected to occlude LTD. Such a depressed baseline would also be expected to increase the attainable magnitude of synaptic potentiation. Indeed it is not uncommon for groups to report profound difficulties in inducing LTD in acute hippocampal slices. One example of such a case is the study of Giese and colleagues (1998) where LTD could not be elicited in either the wild-type or $CaMKII^{T286A}$ mutant animals. It has also been reported that the magnitude of slice LTD increases with time *in vitro* (Bear, 2003); this could reflect such a slicing-induced further removal of synaptic AMPA receptors.

If, as these studies suggest, the slicing procedure can result in the depotentiation and/or the depression of synaptic responses compared to *in vivo* efficacies, then differences in synaptic properties due to the loss of CaMKII-dependent synaptic potentiation in the mutants could potentially be lost subsequent to a slicing-induced plasticity that resets synaptic efficacy to a similar level in both genotypes. A detailed study of synaptic transmission *in vivo* would be able to reveal whether such an effect is happening in wild-type and mutant mice. Alternatively, hippocampi could be perfusion-fixed and morphological correlates of synaptic strength at a large number of synapses within the CA1 region assessed, such as PSD or spine head dimensions (e.g. Toni et al., 2001;Konur et al., 2003;Okamoto et al., 2004;Chen et al., 2004) or the density of synaptic AMPA receptors (e.g. Desmond and Weinberg, 1998;Matsuzaki et al., 2001;Matsuzaki et al., 2004). It has also been suggested that the specific presence of GluR1 subunit-containing AMPA receptors within synapses could be assessed as a signature of recent experience-dependent plasticity having taken place at those inputs (Malinow, 2003).

It should also be noted that for the experiments performed in this thesis, the slicing conditions developed were optimised to minimise the extent of physical trauma and anoxia so that the surface of the slice presented healthy CA1 pyramidal neurones for whole-cell patch-clamping. The specific modifications to the standard slicing procedure used to achieve such slices were not applied in the studies discussed above. Moreover, tests of slice health, like the ability to patch-clamp neurones on the slice surface, were not performed in the experiments described above. Hence, it is a possibility that the slices prepared in this thesis underwent less slicing-induced damage that, in turn,

resulted in substantially fewer or smaller neuronal changes.

4.4 Environmental Enrichment & the Promotion of Physiological Plasticity in Vivo

4.4.1 Standard Housing may not Provide Adequate Experience for the Detection of a CaMKII^{T286A} Phenotype.

Another possible reason for the inability to detect differences in synaptic transmission between the genotypes might be related to possibility that standard laboratory housing conditions do not provide enough opportunity for the formation of hippocampal-dependent memories and thus the modification of CA1 synaptic strengths. Indeed, it has been suggested that mice raised in standard laboratory conditions would be better considered as environmentally-deprived (Wurbel, 2001). Wurbel has also proposed that standard housing conditions could in fact lead to brain physiology that is "maladaptive", that, in turn, may lead to incorrect conclusions about brain organization and function. If data obtained from wild-type mice raised in standard conditions are not always indicative of 'normal' brain physiology and behaviour, but are contaminated with artifacts resulting from restricted brain growth and development, this could also lead to incorrect conclusions gained from studies of transgenic mice about the roles of particular proteins. Moreover, various learning and memory impairments induced by targeted genetic manipulations have even been shown to be rescued following exposure of the animals to enriched environments (Rampon et al., 2000b;Tang et al., 2001b;Martinez-Cue et al., 2002;Tremml et al., 2002). This does not mean that the findings from animals in standard housing are not useful, far from it, but the use of enrichment does directly demonstrate the important ability of the brain to be able to recruit mechanisms that can compensate for the loss of a protein's function when animals are exposed to more stimulating environments. Thus, without the use of the standard housing condition the potential involvement of a protein in, for example, learning and memory might be missed, whereas the use of enrichment has the potential to reveal whether the protein of interest is critical or not for the neural processes and animal behaviours that are under investigation.

Importantly, environmental enrichment does not rescue spatial learning abilities in the

αCaMKII^{T286A} mutant mice, as tested in the Morris water maze (Need and Giese, 2003). This evidence supports the claim that the Thr286 autophosphorylation of αCaMKII is critical for the cognitive processes that underlie certain forms of learning in the murine brain (e.g. Irvine et al., 2005)). An additional test that would strengthen the hypothesis that these forms of learning also require LTP-like forms of synaptic plasticity would be to confirm that LTP remains absent in the enriched αCaMKII^{T286A} mutants. It has also not been assessed whether enrichment results in the up-regulation of the βCaMKII isoform which might be able to compensate for some of the lost CaMKII-dependent functions in the mutant animals. Whether or not enhanced βCaMKII levels exist, we do know, however, that any changes in βCaMKII cannot rescue the processes that underlie hippocampal-dependent learning in the mutant mice, as demonstarted by Need et al. (2003).

4.4.2 The use of Environmental Enrichment to Promote Brain Plasticity In Vivo

To investigate further the neural effects resulting from a lack of αCaMKII-dependent plasticity, environmental enrichment was employed to expose the mice to environments that would encourage them to engage in exploratory and learning activities and to drive the plastic processes that underlie hippocampal αCaMKII-dependent learning and memory. The objective was to increase the probability of detecting differences in synaptic efficacy and/or neuronal structure between the genotypes in order to provide evidence that CaMKII-dependent plasticity can occur as a consequence of enhanced experience.

As discussed in detail within introduction section 1.11, there are many lines of evidence which support this rationale for using environmental enrichment. For instance, molecular investigations have shown that a large number of enrichment-driven changes occur in the hippocampus, suggestive of increased levels of neural activity as well as synaptic and structural plasticity (e.g. Foster et al., 1996;Gagne et al., 1998;Rampon et al., 2000b;Williams et al., 2001;Pinaud et al., 2001;Tang et al., 2001b;Nithianantharajah et al., 2004). Housing animals in enriched environments has also been found to enhance their performance in a variety of hippocampal-dependent behavioural tests of learning

and memory, including the Morris water maze, contextual fear conditioning and novel-object recognition tasks (Paylor et al., 1992;Kempermann et al., 1997;Tees, 1999;Rampon et al., 2000b;Duffy et al., 2001;Williams et al., 2001;Faverjon et al., 2002;Frick and Fernandez, 2003;Need and Giese, 2003) suggesting that the molecular changes initiated by enriched experience may be linked with the enhanced learning capabilities of mice. Furthermore, properties of synaptic transmission and experimentally-induced plasticity have also been found to be altered in hippocampal slices from enriched animals (Green and Greenough, 1986;Foster et al., 1996;Foster and Dumas, 2001;Duffy et al., 2001) suggesting that experience-dependent synaptic changes had indeed occurred in the enriched animals. Overall, the wealth of evidence suggests that environmental enrichment stimulates brain growth and plasticity to occur. Enrichment was therefore used in this thesis in order to increase the probability of detecting differences between the genotypes to order to give clues about the real physiological consequences of αCaMKII-dependent plasticity that occurs *in vivo* within the murine brain.

In the enriched housing condition, the many novel objects, types of bedding material and spatial arrangement of the cage contents were changed at least 5 days a week for the 3 to 5 weeks of enrichment in order to maximize potential for learning and memory to take place. Note however, that differences between the genotypes in their levels of motor activity or their motivation for exploring novel environments were not controlled for, although there has been no evidence to date to suggest that modified αCaMKII function would result in such behavioural differences. Indeed, normal performance of the mutant mice in the visible version the Morris water maze has indicated that the point-mutation does not result in either visual or motor co-ordination impairments that might affect activity levels in the mutant mice (Giese et al., 1998;Need and Giese, 2003). If activity levels in the mutant mice are comparable to the wild-types then it can also be assumed that other changes in brain neurochemistry that may result from increased levels of exercise (Cotman and Berchtold, 2002) would also be comparable between the genotypes.

4.5 Enrichment-Mediated Synaptic & Structural Changes are

Primarily found in αCaMKII^{T286A} Mutant Mice

Contrary to expectations, environmental enrichment did not induce significant changes to the mean values for any of the measures of synaptic efficacy or neuronal structure in wild-type mice, and in only one case (median mEPSC amplitudes) was the the distribution of measuements altered by enrichment. In mutant mice, on the other hand, enrichment resulted in changes to all of the measures of Schaffer collateral evoked transmission, affected the amplitude of mEPSCs and the density of spines in the CA1 apical dendrites.

With respects to the features of evoked transmission, mean EPSC amplitudes (including failures) were significantly greater in the mutant mice that underwent enrichment. The locus of the effect seemed to be largely presynaptic since it was accompanied by a significant reduction in failure rates and the degree of paired-pulse facilitation. These findings suggest that enrichment resulted in an increase in Schaffer collateral release probabilities. However, if it is the case that adult Schaffer collateral axons form multiple synaptic contacts with individual CA1 neurones, as some evidence has suggested (e.g. Isaac et al., 1998;Hsia et al., 1998;Toni et al., 1999;Oertner et al., 2002), fewer failures in the enriched mutants could also arise from the existence of fewer postsynaptic silent synapses (Liao et al., 1995;Liao et al., 2001). However, this by itself would not also cause a change in PPR.

To look at the effect of enrichment at a larger proportion of excitatory inputs to CA1 neurones (irrespective of their origin) recordings of mEPSCs were made. Interestingly, there was no significant increase in the frequency of mEPSCs in the enriched mutant mice, as might be expected when release probabilities are increased. In fact any trend was in the opposite direction with a tendency towards a decrease in frequency. It is possible that if the higher release probabilities are specific to the Schaffer collateral inputs, then changes to the overall mEPSC frequency may be too small to be detected. Alternatively, the lack of an overall increase could be due to compensatory decreases in release properties occurring elsewhere in the neuron (Royer and Pare, 2003) or to an overall decrease in total synapse number. Indeed, in the mutant mice, enrichment was found to cause a significant decrease in CA1 spine density within the proximal/middle regions of the apical dendritic trees – where the majority of excitatory inputs are

thought to arise from Schaffer collaterals axons (Ishizuka et al., 1990;Li et al., 1994).

Almost all excitatory inputs within *stratum radiatum* (and *stratum oriens*) form synapses onto dendritic spines, which generally receive only one synaptic contact (Harris and Stevens, 1989;Schikorski and Stevens, 1997;Megías et al., 2001). Thus a decrease in spine density is likely to reflect a similar decrease in synapse density (assuming that the proportion of synapses forming onto dendritic shafts remains unaltered). Such a decrease could offset the increase in Schaffer collateral release probabilities, resulting, therefore, in no net change in overall mEPSC frequency. No changes in spine density were found in the basal dendrites, which also receive inputs from Schaffer collateral axons as well as recurrent CA1 collaterals (Amaral and Witter, 1989). This shows that the effect of enrichment upon spine/synapse density in the mutant mice was specific to the apical dendrites. As spine density was not assessed in the distal dendrites, it cannot be concluded whether the decrease was specific to dendrites that lie within the stratum radiatum or whether it also applied to dendrites within the stratum lacunosum-moleculare where inputs from the entorhinal cortex and different subcortical structures (e.g. thalami and amygdale) form synapses with CA1 neurones (Amaral and Witter, 1989).

A second effect of enrichment in the mutant neurones was a significant decrease in mEPSC median amplitudes which were on average 14% smaller. It is also possible therefore that any increase in mEPSC frequencies in the enriched mutant cells could have been masked if a higher proportion of events were being lost into the baseline noise due to their reduced peak amplitudes. Indeed, a positive correlation was found between mEPSC amplitude and frequency across cells, suggesting that such a masking effect may have occurred. Importantly, there was no correlation between baseline noise levels and mEPSC amplitudes or frequencies, indicating that differences in experimental recording conditions did not significantly affect the detection of mEPSCs and thus produce this correlation. If it were the case that a higher proportion of events are being lost into the noise in recordings from enriched mutant mice owing to their smaller amplitudes, then the effect of enrichment upon mEPSC amplitude may in fact be underestimated, with only the largest events being detected.

Note, the median mEPSC amplitudes were also reduced by enrichment in the wild-type neurones (on average by 9%) and although the overall reduction was not found to be significant, it was found that the distribution of median mEPSC amplitudes across cells was significantly affected by enrichment such that the values from enriched wild-type mice became more similar.

Enrichment-mediated reductions in mEPSC amplitudes may reflect small postsynaptic decreases in synaptic strength due to decreases in AMPA receptor number and/or receptor efficacy to glutamate. Alternatively postsynaptic responses may in fact have remained stable but the distribution of presynaptic release probabilities changed such that either the probability of small-amplitude mEPSCs (as measured at the soma) increased or that of large-amplitude mEPSCs decreased, thus reducing the median value. This could arise due to changes in release probability at synapses with high versus low postsynaptic efficacy, or due to a change in the relative locations within the dendritic tree from which the majority of mEPSCs arise and thus altering the average affect of amplitude attenuation arising from dendritic filtering. However, the identified phenomenon of 'synaptic scaling' in CA1 neurones would imply that this latter possibility is unlikely. Synaptic scaling is proposed to render the amplitude of a synaptic current, as measured at the soma, *independent* of the dendritic location from which it arises due to mechanisms that compensate for the attenuating effects of dendritic filtering (Stricker et al., 1996;Smith et al., 2003). Some have proposed that the compensatory mechanisms are provided by active dendritic conductances that 'boost' EPSC amplitudes (Lipowsky et al., 1996;Gillessen and Alzheimer, 1997). If this is the case scaling could be disrupted under voltage-clamp conditions, thus differences in the enriched mutants in the dendritic locations that mEPSCs arise could account for differences in median mEPSC amplitudes. However, others have rejected this hypothesis, and propose the synaptic ligand-gated conductances progressively increase with electrical distance from the soma and provide the mechanism of scaling (Stricker et al., 1996;Smith et al., 2003). In this case, differences in the relative locations of synapses that give rise to mEPSCs could not account for differences in median amplitudes.

Moreover, the rise-times of mEPSCs in the mutant mice were not affected by

enrichment. This shows that the reduction in mEPSC amplitudes was not due to an enrichment-associated increase in the levels of dendritic filtering upon mEPSC amplitudes. This also suggests, therefore, that the *average* distance from the soma from which mEPSCs arose was not affected by enrichment (but this does not necessarily mean that the distribution of distances was unaffected).

Overall, these findings show that although CaMKII-dependent potentiation of synaptic strengths is blocked in CA1 neurones of the αCaMKII^{T286A} mutant animals (Giese et al., 1998), plasticity of presynaptic release probabilities and neuronal structure is able to occur via mechanisms that do not require Thr286 autophosphorylated αCaMKII. Postsynaptic deceases in synaptic strength may also have occurred and could account for the enrichment-mediated decrease in mEPSC amplitudes, although presynaptic changes may also have brought about this decrease. The fact that environmental enrichment did result in changes in the mutant mice shows that the enrichment paradigm used was sufficient to drive neural function and expose differences in synaptic and structural properties between wild-type and mutant neurones, whereas housing animals in standard environments was not.

The next section will consider the surprising result that enrichment did not reveal experience-dependent changes in CA1 neuronal properties in the wild-type mice; discussed in the context of results from previous enrichment studies by other groups.

4.5.1 The Stability of CA1 Properties in the Wild-Type Mice in Context of the Findings from Previous Enrichment Studies

If, as hypothesised, enrichment recruits processes that are similar to those required for LTP, then one might have expected to observe increases in the baseline measures of CA1 synaptic strength in the wild-type mice. However, as shown, the mean measures of synaptic strength assessed using minimal stimulation and recordings of mEPSCs remained unchanged. Similarly, measures of spine density were unable to uncover evidence that structural plasticity had occurred in the wild-types. Nevertheless, enrichment did alter both synaptic and structural properties of CA1 neurones in the CaMKII^{T286A} mutant mice indicating that the enrichment paradigm used was sufficient to provoke changes in hippocampal functioning compared to the standard environment

controls. So why were no overall effects of enrichment found in the wild-type mice upon the mean synaptic and structural parameters that were assessed? Is it possible that changes do not occur as a consequence of environment-stimulated increased brain activity? This does not lie in agreement with the many past studies of enrichment. Alternatively, it is possible that changes were simply not detected and that other modes of investigation would be able to reveal the changes brought about by enrichment. Indeed, the finding that the distribution of median mEPSC amplitudes across cells was altered depite the mean median mEPSC amplitudes not showing a significant difference between wild-type neurones from standard and enriched environments, supports the hypothesis that enrichment does result in altered synaptic transmission in the mouse hippocampus and that other lines of investigation are required to detect and quantify the changes.

Receptor binding studies and electrophysiological investigations have both provided evidence to suggest that levels of excitatory transmission are increased after enriched experience. Two studies have shown that environmental enrichment in rats increases the level of ^{3}H-AMPA binding in hippocampal slices by approximately 40% compared to controls that are individually housed in standard cages (Foster et al., 1996;Gagne et al., 1998; animals were enriched for 1-6 hours per day for 4-5 weeks). Gagné et al. report that the increased binding was most likely due to increased redistribution of receptors from intracellular pools (Baude et al., 1994) to the neuronal membrane since the capacity for Ca^{2+} to up-regulate ^{3}H-AMPA binding was significantly decreased in the enriched animals (Gagne et al., 1998) whilst total levels of both AMPA receptor mRNA and protein were unaffected (Foster et al., 1996;Gagne et al., 1998). Such studies cannot, however, differentiate between increased levels of synaptic versus non-synaptic receptors. If redistributed receptors are targeted to synaptic sites, the increase in receptor binding could be due to either an increase in receptor number at individual synapses or an increase in the total number of functional excitatory synapses. In a subsequent study, using identical enrichment procedures, Foster went on to provide evidence that enrichment did in fact result in increased synaptic efficacy at individual synapses (Foster and Dumas, 2001). Their observed increases in synaptic strength were not attributed to changes in presynaptic function as failure rates, paired-pulse ratios and estimates of mean quantal content at unitary inputs remained unaltered. Indeed Foster

and Dumas posed that as the increased synaptic amplitudes were observed in the absence of a change in the coefficient of variation, this provided strong evidence that differences in synaptic efficacy between enriched and control animals were the result of enrichment-mediated postsynaptic changes. Foster and Dumas also found that their estimates of quantal size were greater in the enriched animals supporting this conclusion.

If a redistribution of AMPA receptors into synaptic sites had occurred with enrichment in the wild-type mice of this thesis, I could have expected to observe an increase in minimally evoked EPSC amplitudes as well as mEPSC median amplitudes. Or, if an increase in the number of functional synapses had occurred, then an increase in mEPSC frequency would be expected. So why were neither of these effects found? There is an important difference in the experimental protocol of this study that could account for the differences between the results here and the findings of Gagné and Foster. In addition to their use of rats (thus potential differences due to species), these studies used control rats that were raised in isolated conditions. Isolation rearing is well documented to have many effects upon animal behaviour and neurophysiology (reviewed in Hall, 1998). Effects include increased levels of anxiety (e.g. Wright et al., 1991), impaired spatial learning (e.g. Juraska et al., 1984;Lu et al., 2003b;Hellemans et al., 2004), reduced neurogenesis in the dentate gyrus (Lu et al., 2003b) and altered synaptic transmission and plasticity in various brain regions including the dentate gyrus and hippocampal CA1 region (e.g. Lu et al., 2003b;Bartesaghi, 2004). Thus it is possible that the differences in AMPA receptor binding and synaptic strength seen in the above studies are actually more indicative phenotypes of isolation-rearing in control rats and not necessarily caused by enriched experience. If the enriched rats had been compared to control rats reared in standard social housing, no differences might have been found.

Another study that looked at glutamate receptor levels in enriched mice compared to those found in controls did use social standard housing conditions rather that isolated animals (Tang et al., 2001b). In contrast to the aforementioned studies in rats, this study found that exposure to enriched environments (3 hours per day for 2 weeks) did cause an increase in glutamate receptor protein levels (which the studies of Foster and Gagné did not see), including that of GluR1 (the AMPA receptor subunit that is driven into

synapses during LTP induction; Hayashi et al., 2000) and the NMDA receptor subunits NR2A and NR2B (Tang et al., 2001b). Neither agonist binding experiments nor measures of synaptic transmission or plasticity were assessed in these mice, thus it is unknown whether the increased total protein levels were accompanied by enhanced synaptic strength. The enriched mice did, however, exhibit enhanced learning abilities compared to controls suggesting that changes in neurophysiology had occurred that enhanced cognitive abilities.

There has been no previous evidence, therefore, that has directly shown enrichment to enhance synaptic strength at individual synapses over that found in controls raised in standard social housing. Moreover, another study in mice has provided some evidence that baseline measures of synaptic strength within CA1 in the hippocampus may indeed remain unaffected by enrichment while the animals did exhibit enhanced learning abilities and altered properties of LTP (Duffy et al., 2001). Their measures of baseline transmission came, however, from field recordings (the ratio of field recording EPSP slope to presynaptic fibre volley amplitude) which assess synaptic transmission between many pre- and post-synaptic cells; thus it cannot be ruled out that changes did not occur, for example in the distribution of synaptic strengths or the level of connectivity between particular cell pairs. This can also not be ruled as a possible effect of enrichment in this thesis, as was discussed in relation to possible undetected differences between the wild-type and T286A mutants in standard conditions (see section 4.3.3). Note, however, that Foster and Dumas (2001) did find that enrichment increased the slope of field potentials recorded within the *stratum radiatum* compared to the isolated controls, thus the increases in synaptic efficacy observed at single synapses did correlate with altered field potentials in that instance.

Changes in spine and synapse density have been associated with environmental enrichment in various regions of the brain (see introduction section 1.11.4). With respects to the hippocampus, it has been found that training rats in a complex environment (4 hours a day for 2-3 weeks), where the animals were encouraged to hunt for food and water, resulted in a significant increase in spine density on the basal dendrites of CA1 neurones (Moser et al., 1994b), whilst no net changes were found in the apical dendrites (Moser et al., 1997). Between training session rats were housed in

standard cages in groups of up to 6 rats, whereas control rats were housed either individually or in pairs (these two controls groups showed similar results). Again, such results could reflect an effect of social deprivation rather than that of enrichment, or at least increase the differences between groups. Indeed, isolation rearing has been associated with a specific decrease in synapse number in pyramidal cells of the occipital and visual cortices (Turner and Greenough, 1985;Sirevaag and Greenough, 1987), as well as atrophy of the basal dendrites of pyramidal cells within the hippocampal CA2 region in guinea-pigs (Bartesaghi and Severi, 2004). Interestingly, another study has also reported that a learning paradigm selectively increased spine density on the basal but not on the apical dendrites of CA1 neurones; all animals from this study were raised in isolation (Leuner et al., 2003).

One study in mice has found, however, that environmental enrichment (3 hours per day for 2 months) increased the density of spines on the apical dendrites of CA1 neurones compared to control mice from standard social housing (Rampon et al., 2000b). This observation was supported by electron microscopy analysis of the stratum radiatum where they observed a selective and significant increase in the density of non-perforated axospinous synapses. No changes in spine density, for either apical or basal dendrites, were seen in wild-type neurones of this thesis; in fact, any change favoured a reduction of spines with enrichment in both compartments. However, an important difference in methodologies between that of Rampon et al and those used here for visualizing dendritic spines could potentially account for the difference in results. In the Rampon study, mice were anesthetised and perfusion-fixed before the brains were removed, sectioned and processed using the Golgi impregnation technique to visualise the dendritic spines. In contrast, live neurones in hippocampal acute slices were filled with fluorescent dye and imaged with confocal microscopy in this study. It has been previously shown by the group of Kristen Harris that relative to perfusion-fixed hippocampus, a 40-50% increase in spine number occurs within the *stratum radiatum* of adult hippocampal slices (Kirov et al., 1999). In fact, the spine densities found in this thesis were approximately 100% larger than that of Rampon et al (16.0 spines/10 μm of dendrite compared to 8.1 spines/10 μm). Part of this difference can be attributed to an underestimation of spine density by Rampon et al. Photomicrographs of golgi-impregnated dendrites are opaque and 2-dimentional, whereas confocal images contain

contrast within the image, resulting from difference dye intensities, and 3-dimensional depth that is observed by scrolling through the consecutive z-sections. Thus spines that protrude at an acute angle from the dendritic shaft are easily identified and would be counted in this study while in golgi images they would appear as more ambiguous 'bumps' off the dendrite and would be prone to be missed during spine counting. Spines that lie directly on top or below the dendritic shaft would be missed in golgi images. However, these were also excluded in this thesis and so would not contribute to the differences between this study and that of Rampon et al. (Importantly, inaccuracies in calculated spine density values brought about by the counting technique would apply to all neurones analysed and in all mouse groups assessed within this thesis, and would not therefore contribute to the differences found between mouse groups). Effects of slicing upon spine density could, however, be different in wild-types and mutant neurones if the changes require the actions of autophosphorylated CaMKII.

Harris and colleagues have suggested that it is the large scale reduction in synaptic innervation that ensues following the cutting of axons that triggers the observed increases in spine and synapse number in hippocampal slices (Kirov et al., 1999;Kirov et al., 2004). In the experiments of this thesis, several steps were specifically taken to reduce levels of excitatory transmission within the slice even further in order reduce the detrimental effects of excitotoxicity and anoxia upon slice health. If the loss of excitatory input does indeed trigger synaptogenesis in hippocampal slices, as hypothesised by the lab of Kristen Harris, then this may well explain the significantly higher spine densities observed in this thesis compared to those of Rampon et al (2000).

If it was the case that spine densities were artificially increased in hippocampal slices then any differences in the spine densities in slices from wild-type mice of standard and enriched conditions may have been masked by these processes. However, this possibility is by no means certain since a second comparative study of slice preparation methods found no such increases to occur within a 3 hour time period after slicing (Fiala et al., 2003), even though Kirov et al had found it to occur within the first 2 hours after slice preparation. As a significant difference in mean spine densities was found between standard and enriched mutant animals, this could mean that if increases are occurring, the difference between these two groups may have been initially larger *in*

vivo and thus not completely masked by spine increases due to slicing. It would be interesting to repeat this study using perfusion-fixed wild-type and mutant brains from both the housing conditions to see if similar results are obtained.

Alternatively, differences in mouse age or the enrichment protocols might account for the differences between this and the Rampon et al. (2000b) study. Mice in this study were housed full-time after weaning in the enriched cages where the objects and their arrangement were changed daily. They were 1.5-2 months of age when spine densities were assessed. In the Rampon study, mice were more mature when first introduced to enrichment (1.5-2 months of age), exposed to the novel environments for only 3 hours per day and sacrificed for histology at 3.5-5.5 months of age. It is unlikely that differences in mouse age would account for the 2 fold higher spine densities seen in this thesis compared to those found by Rampon. Dendritic spine loss in hippocampal neurones is associated with old-age (e.g. Nunzi et al., 1987) but not with the transition from adolescence to adulthood (Kirov et al., 2004), thus it seems more likely that the discrepancies are related to tissue preparation rather than age. With respects to the enrichment protocol, perhaps transfer of mice from the home into the enriched environment for relatively short periods, as performed by Rampon, triggers behaviours and consequently physiological changes that are distinct from enrichment of the home environment as imposed in this thesis. Indeed, short-term exposure to novelty could be likened with spaced-training protocols which are widely known to result in better task-learning and long-term memory in animals compared to those that received massed-training (e.g. Spreng et al., 2002). Similarly, spaced electrical stimulation can generate larger and more persistent forms of LTP in hippocampal slices (e.g. Scharf et al., 2002), as well as produce the kinase signals that have been found to be critical for the increase in and long-term maintenance of dendritic filopodia that are specific to spaced repeated stimulation (Wu et al., 2001).

It has also been found that rats will habituate to novel objects when short exposure trials are massed together, whereas rats that receive trails spaced by 24 hours do not habituate and continue to show typical exploratory behaviour (Commins et al., 2003). Additionally, the rats that received the massed-training in this study did not react to a displaced object (i.e. they didn't recognise spatial novelty) whereas spaced-trained rats

did (Commins et al., 2003). The authors suggest that this implies poor encoding of the environment and impaired spatial memory in the massed-trained rats. Although these specific findings are not directly applicable to the comparison of enrichment protocols they do show that different exploratory behaviours can affect learning and memory and thus could play a factor in shaping the consequences of environmental enrichment.

In summary, the data from wild-type animals, as it stands, shows that CA1 spine densities, the properties of individual Schaffer collateral inputs and gross levels of excitatory transmission (as assessed with mEPSC recordings) are not altered by raising mice in enriched environments. Although the lack of an effect upon presynaptic efficacy lies in agreement with the findings from previous studies of enrichment (Foster et al., 1996;Foster and Dumas, 2001), the absence of postsynaptic changes in synaptic strength or spine density does not (Rampon et al., 2000b;Foster and Dumas, 2001). These dissimilarities might reflect differences in the housing conditions of control animals, tissue preparation techniques or enrichment procedures. It is equally possible that the properties of synapses or the level of excitatory input to CA1 neurones is not grossly affected by enrichment in mice but that only their ability to undergo change is altered, as was observed by Duffy et al (2001). Another possibility is that if enrichment-mediated changes occur at some synapses, opposite changes occur at others and result in overall homeostasis of the magnitude of excitatory input to a single cell (discussed further in the next section).

An interesting question would be to investigate how the functional connectivity of hippocampal CA1 neurones in the wild-type hippocampus is affected by enriched experience; for instance, does a divergence in synaptic connectivity between cells occur, such that any one presynaptic neurone becomes functionally connected with more postsynaptic partners? Synaptic and structural plasticity would both be important processes required to carry out such remodeling of hippocampal circuits (and might also therefore be impaired or altered in the $CaMKII^{T286A}$ mice). On the other hand, might presynaptic neurones increase their control over a more select population of postsynaptic target cells through a mechanism of increasing the number of functional synapses that contact a single postsynaptic neurone (as seen by Ninan and Arancio et al., 2004)? The findings of Foster et al (2001) argue against experience resulting in

such a convergence of inputs. They found that estimates of the number of transmission sites were not changed after enrichment.

4.6 Enrichment-Mediated Changes found selectively in the $\alpha CaMKII^{T286A}$ Mutant Mice

The results from these experiments were surprising because a mutation that impairs the function of αCaMKII and at least one form of synaptic plasticity resulted in enrichment-mediated plastic changes to both the synaptic and structural properties of CA1 neurones that were not detected when αCaMKII function was fully intact. So how could the loss of αCaMKII function in the mutant mice result in enrichment-mediated changes and why were these changes (that evidently did not require Thr286 autophosphorylation) not also seen in the wild-type brain? One possibility is that the changes observed in the mutants were actively inhibited or reversed in the wild-type mice via Thr286 autophosphorylation-dependent mechanisms. Alternatively, the increased brain activity driven by enrichment may simply amplify the effects caused by the dys-regulation of mutant CaMKII and its substrate proteins and thus reveal the true mutant phenotype. These possibilities are discussed in the following sections. It must to be also noted that without further investigation one theory cannot yet be favoured over another.

4.6.1 Does αCaMKII Thr286 Autophosphorylation Inhibit Plastic Change?

If some of the functions of Thr286 autophosphorylated CaMKII are inhibitory and in fact prevent certain enrichment-mediated changes in the wild-type animals, then the loss of such functions in the mutant animals would allow changes to occur that do not occur when the enzyme is intact; i.e. the enrichment-mediated plastic changes are un-inhibited in the mutant mice. If this is the case, then it implies that the wild-type actions of autophosphorylated αCaMKII actually inflict a stabilising influence upon hippocampal synaptic properties and that removing its actions results in more labile synapses that are more prone to disruption, especially in enriched environments. However, this scenario seems highly unlikely as all of the known actions of Thr286 autophosphorylated αCaMKII suggest that it is required for the synaptic plasticity in the adult animal, in

particular for the long-term potentiation of synaptic strength (e.g. Malenka et al., 1989;Malinow et al., 1989;Silva et al., 1992b;Pettit et al., 1994;Lledo et al., 1995;Giese et al., 1998).

Furthermore, CaMKII activity itself has been directly linked with enhanced spine motility (Okamoto et al., 2004), activity-dependent filopodial extension and spine formation (Jourdain et al., 2003), the remodeling of dendritic arbours and the increase in synapse number (Fink et al., 2003). CaMKII has not been associated with the inhibition of morphological change in the adult mammalian nervous system; although, during development, the CaMKII-dependent strengthening of function synaptic contacts is thought to limit the elaboration of neuronal processes and underlie the observed CaMKII-dependent stabilisation of dendritic arbours in certain systems (Wu and Cline, 1998;Zou and Cline, 1999). However, in this thesis, the parallel study of wild-type and mutant mice raised in standard environments allows differentiation between the effects of the mutation upon the developing nervous system and its subsequent interaction with enrichment. Since there were no differences between genotypes in the absence of enrichment, the possibility that the morphological differences in the enriched mutants could be attributed to differences in neuronal maturation during development can be excluded.

In summary, CaMKII is regarded as a pivotal enzyme essential for synaptic change, not the inhibition of change (e.g. Lisman et al., 2002), thus it is highly unlikely that the enrichment-mediated changes found in the mutant neurones were due to the loss of CaMKII actions that inhibit plasticity.

4.6.2 *Are Enrichment-Mediated Changes in Synaptic Efficacy Reversed in the Wild-Type Mice?*

A second, and more likely, explanation why the enrichment-mediated changes could be detected in the mutants but not in the wild-types would be if all changes had occurred in the wild-types neurones but were then subsequently reversed, and that these 'reversal processes' were impaired in the mutant mice. In this case, the initial neural responses to enrichment would have included processes that do not require the autophosphorylation of αCaMKII at Thr286. If these changes are then actively reversed via mechanisms that

do require Thr286 autophosphorylation, then the original changes would not be found in the wild-types but would persist in the mutant mice.

The concept that experience-mediated changes in hippocampal synaptic physiology are only temporary is not new. Synaptic plasticity in the hippocampus is thought to be necessary for the processing of neural information in a way that is required for learning and the formation of long-term memories, but the hippocampus is it not generally considered to provide the substrate for their final storage (e.g. Squire, 1992; but see Cipolotti et al., 2001), although it has been suggested that long-term stable changes in synaptic strength may be important for the long-term stability of hippocampal place-fields in rats (Thompson and Best, 1990;Lever et al., 2002) necessary for the generation/updating of spatial reference frames used by rodents for spatial navigation (McNaughton et al., 1996).

It has long been proposed that memory traces may be permanently stored within regions of the neocortex (e.g. Squire, 1992;Eichenbaum et al., 1996;Rolls, 1996); possibly the regions that are themselves involved in their initial perception. The strongest and most well-known evidence supporting the role of the hippocampus in long-term memory formation but not for long-term storage comes from the study of human patient H.M. who underwent a bilateral medial temporal lobectomy in order to control his intractable epilepsy (Scoville and Milner, 1957;Corkin, 2002). Following surgery, H.M. could still form short-term memories but he lost the ability to acquire new long-term episodic knowledge (memory containing specific spatial and temporal context of an experience) and semantic knowledge (general knowledge about the world). His ability to recollect declarative memories (conscious recall of facts and events) that were formed prior to his surgery remained largely in tact. Thus, the studies of H.M. (and other similar patients) were pivotal in recognizing the hippocampus as a structure necessary for the process of encoding newly acquired information into long-term memory traces but that their final storage and subsequent recollection did not dependent upon its function. Such a job description for the hippocampus has largely been supported by lesion and behavioural studies carried out in rodents, as well as other species (e.g. Nadel and Moscovitch, 1997;Colombo and Broadbent, 2000). Note, however, that a recent clinical study (Cipolotti et al., 2001) has provided evidence that controversially suggests that the

hippocampus also plays a more direct role in accessing/storing long-term memories than was previously thought (Nadel and Moscovitch, 2001;Cipolotti et al., 2001); this proposal is based on the study of a patient that shows a devastating retrograde amnesia after suffering discrete damage to the hippocampal formation while the adjacent entorhinal cortex and temporal lobe are spared, thus permitting conclusions to be drawn about the specific impact of lesions to the hippocampal formation. Their findings are also supported by studies that have shown equally robust activation of the hippocampus during the retrieval of remote memories as compared to that when recent one are retrieved (as detected by functional magnetic resonance imaging; Ryan et al., 2001;Maguire, 2001).

In context of the enriched wild-type mice, the lack of any gross changes in synaptic strength and spine density could be explained by the hypothesis that once memories (formed as a consequence of enrichment) are transferred and permanently stored in brain regions outside of the hippocampus, the CA1 inputs that had undergone modification were then reset/reversed to their baseline states in order to enable their potential reuse in future hippocampal processing and memory formation. Indeed it has been proposed that synapses operate within a 'synaptic modification range' with a ceiling and floor level of synaptic potentiation and depression and evidence from the cortex has suggested that this range is not shifted or expanded in order to accommodate further changes (Rioult-Pedotti et al., 2000). Hence, without a reversal mechanism the hippocampus would quickly become saturated with 'used' synapses unable to undergo further modification and thus hippocampal-dependent learning would become impaired. Indeed it has been shown that saturating synaptic efficacy *in vivo*, using LTP-inducing stimuli, before exposure to a learning task, can interfere with hippocampus-dependent learning and the acquisition of spatial memories (McNaughton et al., 1986;Castro et al., 1989;Barnes et al., 1994;Moser and Moser, 1999). In addition to supporting the hypothesis that the modification of synaptic strength is required for learning and memory formation, this finding also supports the notion that mechanisms must exist to prevent the accumulation of experience-dependent synaptic changes in order to allow further learning to occur.

As discussed in the introduction of this thesis, the two primary models of homosynaptic

activity-dependent synaptic plasticity found in the hippocampus (as well as other brain regions) are LTP and LTD. Both these models of plasticity fulfill the criteria that are considered essential for a potential neural mechanism that could underlie learning and memory in the brain. They are both: input specific (Bliss and Lomo, 1973;Dudek and Bear, 1992), associative and cooperative (McNaughton et al., 1978;Levy and Steward, 1979;Stanton and Sejnowski, 1989;D'Alcantara et al., 2003) and can persist for prolonged periods of time (Bliss and Lomo, 1973;Manahan-Vaughan and Braunewell, 1999). Thus, although LTP has received the most intensive investigation into its potential role in learning and memory, LTD is an equally deserving candidate.

An important role of the hippocampus has been attributed to the encoding of spatial information, thus, plasticity of hippocampal synapses is thought to be particularly important for the formation of spatial memories (O'Keefe, 1999;Jeffery and Hayman, 2004). However, it has not been possible to detect real-time experience-induced synaptic plasticity *in vivo* as animals are exploring novel environments (e.g. Hargreaves et al., 1990;Erickson et al., 1993;Moser et al., 1994a); this may be due to a sparse and widespread distribution of synapses that undergo experience-induced plasticity at any one time or perhaps due to equal amounts of potentiation and depression occurring contemporarily resulting in no net changes taking place. However, it has been shown that allowing animals to explore novel environments can great facilitate the ability to experimentally induce either LTD or LTP within the CA1 region of the hippocampus using electrical stimuli; this facilitating phenomenon was not observed as animals explored familiar environments (Manahan-Vaughan and Braunewell, 1999;Li et al., 2003;Kemp and Manahan-Vaughan, 2004;Davis et al., 2004). This suggests that exposure to novel spatial situations and stimuli can alter the hippocampal neuronal state in a way that favours the modification of synaptic strengths.

Recent evidence has also been presented indicating that the enhanced ability to induce either LTP or LTD in the CA1 region of the rat hippocampus is specifically associated with distinct environmental stimuli. The enhanced ability to induce LTD was found to be associated with the spatial mapping of novel objects to specific locations but not with spatial exploration *per se*; in contrast, spatial exploration, but not object exploration, was specifically associated with the facilitation of LTP induction (Kemp and Manahan-

Vaughan, 2004). Thus, the strengthening and depression of synaptic strengths may differentially encode distinct aspects of novelty acquisition (Kemp and Manahan-Vaughan, 2004).

Furthermore, the selective loss of LTD at CA1 synapses in a strain of PP2B knockout animals, that did not impair the ability of mice to form long-lived spatial reference memories (assessed using the standard Morris water maze paradigm) was found to correlate with specific impairments in behavioural tests of working episodic-like memory (where the animals were required to learn a series of successive novel locations in a water maze; Zeng et al., 2001). LTP and depotentiation at CA1 inputs were intact in these animals. These data suggest that LTD may also be particularly important for the ability of animals to exhibit flexible learning of novel information, a process that may be particularly relevant for animals that are subject to environmental enrichment procedures.

If the findings from these aforementioned studies are physiologically relevant, the strengthening and weakening of hippocampal synapses are likely to occur in parallel (underlying distinct aspects of hippocampal function) as the wild-type mice of this thesis explore their daily enriched environments. However, based upon the proposed concept that all bidirectional plastic changes require continual reversal in order to avoid hippocampal saturation, any evidence of experience-induced synaptic changes in wild-type CA1 neurones could have been erased. Theoretically, the experience-dependent strengthening of synaptic efficacies would be followed by depotentiation, and inputs that encode information through the selective weakening of synaptic contacts would require subsequent potentiation in order to be returned to their initial baseline levels.

In the αCaMKII^{T286A} mutant mice, although hippocampal plastic changes requiring CaMKII-dependent synaptic potentiation, like LTP, as a neural substrate would be inhibited, other intact forms of experience-dependent plasticity that are independent of αCaMKII activity, for instance LTD, would remain intact and could, therefore, occur as the mutant mice explore their enriched habitats. However, if the processes that reverse depressed synapses back to baseline are impaired, these modified synapses would remain in their experience-mediated depressed states and would most likely accumulate

in the mutant CA1 neurones.

The finding that mEPSC amplitudes and spine densities were decreased in the enriched mutant mice supports this hypothesis. The net removal of postsynaptic AMPA receptors by internalisation, a key process underlying the expression of LTD in CA1 neurones (Carroll et al., 1999;Heynen et al., 2000;Beattie et al., 2000;Brown et al., 2005), could result in the observed decrease in mEPSC amplitudes. Furthermore, spine shrinkage (Zhou et al., 2004), spine retraction (Nägerl et al., 2004) and synapse elimination (Shinoda et al., 2005) have all been shown to occur as a result of experimental stimuli that induce LTD. These processes could account for the decrease in CA1 spine densities as well as the trend towards a decrease in mEPSC frequency.

The evidence that enrichment also increased presynaptic release probabilities at mutant CA1 synapses is not, however, indicative of a depressed synaptic state; a higher release probability enhances the chance that an action potential results in synaptic transmission, thus increasing synaptic efficacy. One possibility is that as the number of depressed inputs accumulated in CA1 mutant neurones, changes occurred within the Schaffer collateral terminals (and not necessarily only there) to increase the overall level of excitatory input and CA1 activity as a mechanism to compensate for the loss of LTD-reversal. Therefore, rather than these pre-synaptic changes being a primary effect of enrichment, they may actually reflect a form of synaptic homeostatic plasticity (e.g. Turrigiano, 1999;Turrigiano and Nelson, 2000;Davis and Bezprozvanny, 2001;Royer and Pare, 2003). Importantly, this result also showed that physiological increases in release probability can occur via mechanisms that are independent of Thr286 autophosphorylated αCaMKII.

On the other hand, it may also be the case that this increase in presynaptic function is a primary effect of enrichment, that again cannot be reversed and thus remains and is detected in the mutants. This would indicate that the reversal of increased presynaptic efficacy requires αCaMKII Thr286 autophosphorylation. A number of studies have shown that αCaMKII can interact with various elements of the presynaptic vesicular release machinery, including synapsin I and syntaxin 1A (e.g. Nayak et al., 1996;Ohyama et al., 2002). Thus it is feasible that CaMKII may play a role in mediating

presynaptic changes in synaptic efficacy. However, presynaptic actions of CaMKII have so far only been associated with increased presynaptic function and not with the depression of presynaptic efficacy (e.g. Nayak et al., 1996;Ninan and Arancio, 2004). Evidence from one study has indicated however, that CaMKII may exert a negative influence upon the short-term facilitation of presynaptic function (Hinds et al., 2003); thus it is may be possible that disruption of a CaMKII-dependent constraint upon vesicle release actually leads to a long-term facilitation of presynaptic function in the enriched mutant animals.

4.6.2.1 Synaptic Depression in CaMKII^{T286A} Mutant Mice

One crucial aspect of this 'reversal' hypothesis that has not been demonstrated however, is whether mechanisms of homosynaptic synaptic depression (e.g. LTD) remain intact in the CaMKII^{T286A} mutant mice. In the initial study of these transgenic mice, the authors were unable to demonstrate experimental LTD in either wild-type or mutant hippocampal brain slices (Giese et al., 1998); this complies with the notorious reputation that LTD holds for being difficult to induce, being sensitive to both animal age and experimental conditions (e.g. Kemp et al., 2000). As discussed earlier, this reported inability to induce LTD in brain slices could in fact reflect the occlusion of synaptic depression, resulting from a slicing-induced dephosphorylation of AMPA receptors at the PKA phosphorylation site Ser845 (Ho et al., 2004); a key process known to precede AMPA receptor internalisation and LTD induction (Ehlers, 2000).

Based on the current knowledge of the mechanisms that underlie LTD at CA1 synapses (recent reviews include: Kemp and Bashir, 2001;Malinow and Malenka, 2002;Malenka and Bear, 2004), the loss of αCaMKII autophosphorylation is unlikely to impair LTD expression in the mutant mice. Like LTP, the initial signal for the induction of LTD is a postsynaptic rise in calcium concentration, however, the magnitude of the calcium influx is such that protein phosphatase cascades are preferentially activated over protein kinases (e.g. Mulkey et al., 1993;Mulkey et al., 1994;D'Alcantara et al., 2003). With respects to CaMKII and the Ca^{2+}/CaM-activated phosphatase protein phosphatase 2B (PP2B), the preferential activation of PP2B during LTD induction is thought to occur due to its higher affinity for Ca^{2+}/CaM over that of inactive CaMKII subunits (Winder and Sweatt, 2001;Mansuy, 2003;Xia and Storm, 2005). Furthermore, any residual

CaMKII activity would itself be inhibited during increased PP2B activity due to the dephosphorylation of Thr286 by protein phosphatase 1 whose activity is also up-regulated as a consequence of PP2B activation (Lisman, 1989). Indeed, LTD was reported to be unimpaired at CA1 synapses in hippocampal slices when induced in the presence of a peptide inhibitor of CaMKII activity (Chen et al., 2001). Moreover, in keeping with the proposal that CaMKII activity is in fact inhibited rather than promoted during LTD, the application of the CaMKII inhibitor KN-62 has been shown to facilitate LTD in hippocampal CA1 neurones (Schnabel et al., 1999).

In contrast, an earlier study found that the inhibition of CaMKII by bath application of KN-62 blocked synaptically-induced LTD in hippocampal slices from P21 rat, implicating a requirement in this case for CaMKII in LTD induction (Stanton and Gage, 1996). The authors concluded that this experimental form of LTD must act through the reduction in presynaptic glutamate release via a mechanism dependent upon presynaptic CaMKII activity since postsynaptic infusion of the CaMKII inhibitor did not block LTD induction. However, a more recent study found that while a form of CA1 LTD in neonatal animals is mediated by large decreases in presynaptic function, a developmental switch in the expression mechanisms occurs such that little or no change in presynaptic function at CA1 synapses occurs in the hippocampus of rats aged 21 days and older (Nosyreva and Huber, 2005). This agrees with the conclusions of other studies that LTD in adult animals is mediated largely by postsynaptic mechanisms that modify AMPA receptor transmission (Malinow and Malenka, 2002). Furthermore, although pre-synaptic CaMKII activity is known to be involved in the regulation of vesicle mobilization through the phosphorylation of synapsin I (Sihra et al., 1989;Chi et al., 2001), it is the *de*-phosphorylation of synapsins by phosphatases that has been found to impose inhibitory constraints upon evoked transmitter release (Jovanovic et al., 2001). Thus an explanation for the findings of Stanton and Gage (1996) remains elusive.

Importantly, LTD has been shown to be intact in brain slices from αCaMKII knockout mice; both in the visual cortex (Kirkwood et al., 1997) and the CA1 region of the hippocampus (Stevens et al., 1994), although the magnitude of LTD was reported to be diminished in some hippocampal slices. The demonstration of intact synaptic LTD in the $\alpha CaMKII^{T286A}$ mice is still a very important experiment that remains to be

performed in order to be able to promote the idea that the experience-mediated synaptic and structural changes observed in the mutant mice could have resulted from activity-induced synaptic depression. Nonetheless, based on all the evidence discussed above, it is still reasonable to assume that LTD in adult mice *in vivo* does not require CaMKII and would be intact therefore in the $CaMKII^{T286A}$ mutant mice. Thus the enrichment-mediated decreases in mEPSC amplitudes and spine densities found in the mutants could potentially be the result of experience-dependent depression of synaptic strengths. The observation that a decrease in spine density was restricted to the apical dendrites could also be interpreted as suggesting that experience-induced synaptic depression may not have occurred in the basal dendrites of CA1 neurones; a finding that probably reflects differential functions of the synapses within the basal and apical dendritic arbours.

4.6.2.2 Evidence from other Studies and Hypothesised Roles of Plasticity Reversal

In support of the hypothesis that intact plastic mechanisms in the wild-type mice would prevent the accumulation of modified synapses in the hippocampus, electrically-stimulated synaptic potentiation (i.e. experimental LTP) induced in the hippocampus (Xu et al., 1998) and in dentate gyrus (Abraham et al., 2002;Abraham et al., 2003) of freely moving rats was shown to be reversed when the animals enter and explore a novel environment. These studies show that inherent mechanisms are likely to exist *in vivo* that constantly drive synapses towards their default baseline strengths and prevent large net changes in synaptic weighting persisting in hippocampal neurones.

Xu et al (1998) showed that the reversal of LTP in the CA1 region was: a) specific to the potentiated inputs, as the ipsilateral control pathways remained stable, b) persistent, thus not simply a transient activity state-dependent form of depression, and c) specific to the experience of novelty, when the rats were familiarised to the novel environment during a 1 hour exposure on two consecutive days, re-exposure on the following day 1 hour after LTP induction now failed to reverse LTP. They also found that novelty correlated with increased hippocampal activity with a dominant frequency of 6-8 Hz. This lies within the theta frequency range (5-10 Hz) that has previously been identified as an optimal stimulation range for inducing experimental depotentiation of recently established LTP (e.g. Huerta and Lisman, 1995;Stäubli and Chun, 1996;Doyle et al.,

1997). Thus the observed novelty-induced reversal may share mechanisms with electrically-induced depotentiation (e.g. Huang et al., 2001). In keeping with this proposal, the recently familiarised environments (i.e. those that were previously novel) failed to promote the occurrence of theta activity in the rat hippocampus as well as failing to reverse LTP (Xu et al., 1998). It would be interesting to see whether *in vivo* brain activity driven by the exposure to novelty would also cause the reversal of experimentally-induced LTD in the hippocampus.

Furthermore, hippocampal theta oscillations induced *in vitro* by the application of cholinergic agonists have been found to heighten slice sensitivity to bi-directional plastic changes induced experimentally (e.g. Huerta, 1993;Huerta and Lisman, 1995;Huerta and Lisman, 1996). Thus it is likely that the observed facilitation of LTP and LTD in rats during their exposure to particular novel environmental stimuli (Manahan-Vaughan and Braunewell, 1999;Li et al., 2003;Kemp and Manahan-Vaughan, 2004) results from this novelty driven increase in hippocampal theta activity (Xu et al., 1998).

During exposure to novel stimuli, synaptic input to the hippocampus may, therefore, fulfill two distinct roles: 1) the delivery of informative neural code (hypothesised to be in the form of correlated pre- and postsynaptic activity to induce homosynaptic plasticity (Hebb, 1949) that is processed by the hippocampus into memory traces, and 2) to simultaneously stimulate other synapses to reverse towards their default baseline strengths. The role of this simultaneous reversal may be to prevent the stabilisation of synaptic modifications that result from inconsequent or incidental correlated activity, thus reducing error and the possible scrambling of synaptic networks (Zhou and Poo, 2004). It could also serve to erase previously formed memory traces that have become labile after their transfer to the neocortex. In this case, synapses that undergo information laden plasticity must also possess active processes to resist this reversal until the appropriate time (Zhou and Poo, 2004).

In the study of Xu et al (1998), brain activity initiated by the rats' experiences in novel environments was found to be sufficient to reverse the recently induced experimental LTP; however, under physiological conditions, other forms/magnitudes of synaptic drive or different neuronal states may also be important for reversing temporary

experience-induced changes at hippocampal synapses (or, alternatively, consolidating them). For instance, it has been proposed that brain activity that occurs during sleep is important for the consolidation of memory traces in the brain (Walker and Stickgold, 2004). Such sleep-mediated plasticity could, theoretically, also be important therefore for re-establishing equilibrium of hippocampal synaptic weights following the consolidation of synapses and storage of memory elsewhere in the brain.

Xu et al (1998) also found that exposure to novel environments was not sufficient to reverse potentiated CA1 inputs if the rats were only introduced to them 24 hours after LTP induction. Such rapid novelty-induced reversal only seems relevant, therefore, for unconsolidated synapses. Abraham and colleagues have similarly observed that novel environments provoke the reversal of experimental LTP in the dentate gyrus of freely moving rats; however, the time window for LTP reversal by experience was much longer, lasting for at least 14 days (Abraham et al., 2002;Abraham et al., 2003). These differences could reflect either distinct physiology of the pathways studied, rat strain differences or differences in their LTP induction protocols used.

Physiologically, perhaps this proposed form of experience-driven reversal that occurs within a relatively short time-window could reflect a mode for resetting synapses that have undergone previous modification as a consequence of the hippocampal *processing*, but <u>not</u> as a mechanism for erasing plasticity at synapses that contribute towards *memory traces* – which as mentioned above must be able to resist this reversal until consolidated elsewhere. Indeed, evidence has suggested that the proportion of hippocampal CA1 neurones that are activated during exposure to a novel environment may be as high as 40% (Guzowski et al., 1999;Vazdarjanova and Guzowski, 2004). Thus the proportion of synapses that undergo activity-dependent modification during the encoding of neural information could also be as large, potentially rendering this form of reversal crucial for the maintenance of processing capacity in the hippocampus. On the other hand, the proportion of synapses that are modified and contribute towards actual memory traces may be comparably small. Once a memory trace *is* permanently stored outside the hippocampus, changes may then ensue that do permit the resetting of these synapses; either by a distinct mechanism of reversal or via a change that removes their previous protection from reversal, yielding them labile and thus susceptible to this

proposed action of theta-frequency brain activity.

It is also important to stress the point that the experiments described above observed the reversal of experimental LTP, and not the reversal of synapses that have undergone physiological modification. Thus, reversal in these preparations may simply reflect the ability of the brain to re-establish synaptic equilibrium after experimental interference. In the brain of laboratory rats housed in simple environments, the synaptic drive to CA1 neurones may be insufficient to maintain homeostasis, but the activity acquired through exposure to novelty may be able 'kick-start' the neurones and enable them to reverse the non-physiological synaptic changes that were exogenously induced via electrical stimulation. If the animals are deprived of stimulation for longer periods of time however, the aberrant changes may indeed undergo a form of consolidation and thus become more difficult to reverse, but this may not necessarily reflect the ability of consolidated memories to remain in the hippocampus for prolonged periods of time.

4.6.2.3 A Possible αCaMKII-Dependent LTD Reversal Mechanism

As discussed above, it is likely that the processing of neural code and the formation of hippocampal memory traces recruits mechanisms of synaptic weakening as well synaptic strengthening. The loss of CaMKII-dependent processes that reverse depressed synapses could therefore explain the apparent enrichment-mediated synaptic depression that persists in the $\alpha CaMKII^{T286A}$ mutant animals. The most obvious manner by which synaptic depression might be reversed would be via the mechanisms of activity-dependent potentiation; the source of synaptic activity potentially driven by increased theta activity, generated, for instance, through altered states of arousal or by particular stages of sleep.

Indeed the major field of research into neuronal CaMKII has studied the mechanisms by which activated CaMKII increases synaptic strength (e.g. Rongo, 2002;Griesbach et al., 2004 and see introduction section 1.7.3) and see introduction section 1.73. For example, synaptic efficacy can be increased by the phosphorylation of AMPA receptors by CaMKII at residue Ser831 which enhances channel function by increasing the probability that the channel makes transitions to its high-conductance states (Derkach et al., 1999;Derkach, 2003). CaMKII is also required for the activity-dependent

recruitment of additional AMPA receptors into synapses (Hayashi et al., 2000;Andrasfalvy and Magee, 2004), although the precise mechanisms of this process remain unsolved.

Long-term increases in synaptic strength have also been associated with changes in the morphological features of dendritic spines and synapses. Noted changes include increases in synaptic contact area (Desmond and Levy, 1986;Toni et al., 2001), rapid changes in spine and filopodia length following LTP induction (Maletic-Savatic et al., 1999;Yuste and Bonhoeffer, 2001) and the appearance of new spines (Engert and Bonhoeffer, 1999;Jourdain et al., 2003;Nägerl et al., 2004), although it has not been demonstrated that long-term changes in spine/synapse number are necessary or for the maintenance of LTP. With respects to this thesis, if the reversal of synaptic depression via mechanisms of synaptic potentiation is also accompanied by correlative increases in spine density in the wild-type mice this could also explain the lack of detectable changes in CA1 spine density in the wild-type animals and the persistence of reduced spine densities in the mutants.

Such reversal-mediated increases in spine density would be blocked if they require the actions of autophosphorylated/autonomous CaMKII holoenzymes directly or if they are triggered by other downstream effectors that result from CaMKII-dependent increases in synaptic efficacy. Although specific mechanisms have not been resolved, recent evidence has shown that experimentally increasing CaMKII activity (achieved by either injecting or over-expressing the kinase or calmodulin, or by blocking phosphatase activity) results in increased synaptogenesis in cultured hippocampal neurones (Fink et al., 2003) and filopodial growth and spine formation in organotypic cultures (Jourdain et al., 2003). These authors propose that these processes may be mediated through the ability of the βCaMKII-containing holoenzymes to bind to actin (Shen and Meyer, 1999) which serves a cytoskeletal role and provides a scaffold for various synaptic proteins within dendritic spines (e.g. Sheng and Pak, 2000). Indeed the interactions and dynamics of the actin cytoskeleton have been intensely studied in recent years and it is recognised as playing a critical role in the plasticity of spine morphology and physiology (e.g. Matus, 2000;Rao and Craig, 2000;Zhou et al., 2001;Star et al., 2002;Penzes et al., 2003;Hering and Sheng, 2003). It was recently demonstrated, using

a technique that can directly image the dynamics of actin polymerization, that specific increases in actin polymerization within spine heads can be triggered by tetanic stimulation and the activation of NMDA receptors. Such an increase in spine head actin content, in turn, increases the spine's binding capacity for CaMKII holoenzymes thereby targeting more CaMKII to the potentiated sites (Okamoto et al., 2004). Moreover, long-lasting actin- and activity-dependent increases in spine head dimensions and efficacy have been shown to require CaMKII activity (Matsuzaki et al., 2004) further supporting a critical role of the kinase in morphological as well as synaptic plasticity.

In addition to the possibility that activity- and CaMKII-dependent 'LTP-like' phenomena could be responsible for reversing experience-dependent decreases in spine densities (the potential structural correlates resulting from synaptic depression) in the wild-type mouse brain, it has recently been proposed by Fink and colleagues (2003) that CaMKII may also stimulate synaptogenesis in CA1 neurones via an activity-independent 'homeostatic' function of the kinase – a function that would be specifically recruited during periods of prolonged depressed excitatory activity that acts to maintain neurones within an optimal window of excitatory input. Their hypothesis offers an alternative mode by which enrichment-mediated decreases in spine density could be reversed in wild-type mice but persist in the $CaMKII^{T286A}$ mutant mice.

Their hypothesis was based on the observations that specific increases in βCaMKII activity enhanced neurite motility and extension and increased dendrite arborisation and synapse number in cultures of hippocampal neurones (Fink et al., 2003). Although this work was primarily performed on neonatal neurones, blockade of βCaMKII in mature neurones also reduced filopodia motility suggesting that a critical morphogenic role may also exist during adulthood (Fink et al., 2003). They proposed that this would be sufficient for the remodeling of existing arbours in mature neurones, enabling them to maintain their level of excitatory input within a desired window.

Although the mechanism of this CaMKII-dependent process remains unknown, the ability of the β-subunit to bind α/β-holoenzymes to the actin cytoskeleton was found to be critical. They suggested that basal levels or small increases in Ca^{2+} concentration

would be required to trigger such morphogenic actions of the kinase and that it represents a distinct function of the kinase to the more widely studied effects of high levels of postsynaptic Ca^{2+} influx and the triggering of the CaMKII-dependent processes specifically involved in the rapid potentiation of existing synapses. High Ca^{2+} influxes promote the dissociation of CaMKII holoenzymes away from the actin structures and their translocation to the PSD. This is due to the competition between Ca^{2+}/CaM and actin for association to the kinase (Shen and Meyer, 1999); hence the requirement for smaller Ca^{2+} signals that would not result in release of the actin-tethered β-subunits or, therefore, CaMKII translocation.

So how might enrichment-mediated synaptic depression and spine/synapse loss be able to recruit/promote such a homeostatic action of CaMKII? It had previously been shown that reduced levels of excitatory input changes the expression ratio of the α- and β-isoforms such that the relative expression βCaMKII is increased and that of αCaMKII decreased (Thiagarajan et al., 2002). The β-isoform has a significantly higher affinity for Ca^{2+}/CaM than the α-isoform (Hanson and Schulman, 1992) and it has been shown that a βCaMKII rich holoenzyme yields more kinase activity in response to a sub-maximal calcium stimulus than αCaMKII rich holoenzymes (De Koninck and Schulman, 1998). Therefore, an increase in the proportion of βCaMKII subunits could enhance the morphogenic actions of the holoenzyme under basal conditions as was seen by Fink et al when the isoform was overexpressed in cultured neurones. If it is the case that the morphogenic actions of actin-bound CaMKII holoenzymes are impaired in the $\alpha CaMKII^{T286A}$ mutant mice, then this would also suggest that αCaMKII autophosphorylation is also required for such a function. Indeed, since the kinase activity will be low under conditions of basal/moderate Ca^{2+} concentration, inter-subunit autophosphorylation at Thr286 and Thr287, on the respective α- and β-subunits, may be crucial, or at the very least a limiting factor for generating the level of kinase activity required for promoting the morphogenic actions of its substrate proteins. It was previously found that as much as 25% of Thr286/287 is autophosphorylated under basal conditions (Molloy and Kennedy, 1991), thus the T286A mutation could substantially reduce this activity and thus bring the level of kinase activity below the critical level required for a structural recovery from synaptic depression.

The enhancement of this βCaMKII-dependent hypothesised function of the kinase would require a period of time to be initiated/enhanced if it requires the increased translation of βCaMKII mRNA which, unlike the α-isoform, is restricted to the cell soma (Burgin et al., 1990). New β-rich holoenzyme would then also require time to translocate into the dendrites. This supports the proposal that CaMKII could provide a delayed homeostatic cellular response to depression (Fink et al., 2003). Such a CaMKII-mediated form of homeostatic plasticity could underlie the maintenance of spine number in the wild-types and persistence of decreased spine densities in the mutant mice.

4.6.2.4 A Reconsideration of the Mutant Mouse Behavioural Phenotype

Although, learning is severely impaired in mutant mice, some of their residual learning capabilities could be explained by processes that require LTD. However, the accumulation of depressed synapses within the hippocampus in the absence of potentiating reversal mechanisms could also result in the loss of cognitive processing that required this form of plasticity, such as the spatial mapping of novel objects within an environment (Kemp and Manahan-Vaughan, 2004). Thus the etiology of the impaired learning phenotype in the mutant mice could reflect the loss of two distinct processes: 1) the loss of hippocampal functions that require LTP-like processes to lay down neural code, and 2) the loss of hippocampal functions that rely upon the weakening of specific synaptic inputs – due to the irreversible saturation of LTD-like phenomena.

Previous studies and discussions of these mutant mice have not considered the possibility that a reason why spatial learning is impaired could be due to the limited potential of synaptic connections to undergo depression and that when the floor of the synaptic modification range is reached, it is the inability to recovery from depression that may impair cognitive function and thus learning. This is quite distinct from assuming that the animals do not possess the mechanisms required for appropriate hippocampal processing and the laying down of memory traces at all.

4.6.3 Do the Enrichment-Mediated Changes Found in the Mutant Mice Simply Reflect the Consequences of Increased αCaMKII^{T286A}

Dysfunction – Effects that would not occur in Wild-Type Brains?

It is possible that changes found in the enriched mutant animals do not reflect normal physiological consequences of enrichment (as proposed and discussed above) but they might simply result from an enrichment-stimulated increase in aberrant consequences that result from the loss of αCaMKII Thr286 autophosphorylation; i.e. effects that would not occur in wild-type animals and do not reflect normal neuronal responses to enrichment. Indeed, trying to differentiate between mutant phenotypes that reflect the true consequences of a loss of protein function and those that are knock-on effects of mal-development or dys-regulation of a system is an inherent problem in the study of transgenic mice. The detection of such aberrant phenotypes in the enriched mutants but not in those from standard housing most likely means that they are increased in an activity-dependent manner.

If it is the case that the changes seen in the enriched mutants are changes that would never occur in wild-type animals and can be described therefore as 'non-physiological' effects of the mutation, then through what mechanisms could such effects arise? They are two obvious explanations. The changes could have arisen from either 1) the direct dys-regulation of CaMKII-substrates and/or binding partners, or 2) from the altered activity of other Ca^{2+}/CaM-activated proteins due to changes in the CaM availability in the mutant mice. Changes in free Ca^{2+}/CaM might arise because when αCaMKII becomes autophosphorylated at residue Thr286, its affinity for bound Ca^{2+}/CaM increases by 1000-fold (known as CaM-trapping; Meyer et al., 1992) and this slows the release of CaMKII-bound CaM from less than a second to several hundred seconds (Meyer et al., 1992). The loss of this activity-dependent sink for CaM in the $\alpha CaMKII^{T286A}$ mutant animals could, therefore, lead to significant increases in the availability of Ca^{2+}/CaM and hence the inappropriate activation of other Ca^{2+}/CaM-dependent proteins. Such proteins could include PP2B, nitric oxide synthase, adenylyl cyclase 1 and 8 or Ca^{2+} channels. The altered regulation of any of these molecules could mediate plastic changes in synaptic efficacy. Various cytoskeletal molecules are also regulated by Ca^{2+}/CaM (e.g. tubulin, spectrin and microtubule-associated protein 2), thus increased free Ca^{2+}/CaM could potentially result in structural changes in mutant CA1 neurones. The large number of Ca^{2+}/CaM-stimulated/modulated molecules

together with the intricacy of the systems that control their activities as well as those that control the levels of free CaM in neurones (recent reviews include: Chin and Means, 2000;Kortvely and Gulya, 2004;Xia and Storm, 2005) make it exceptionally difficult to predict what the outcome of the loss of αCaMKII-dependent CaM-trapping would be in the mutants. Nevertheless, possible routes by which some of these molecules could have resulted in the enrichment-mediated changes seen in the mutant CA1 neurones are discussed below.

The inappropriate increase in Ca^{2+}/CaM-stimulated PP2B activity under conditions of increased Ca^{2+} influx in mutant neurones could tip the kinase-phosphatase equilibrium in favour of phosphatase activity and promote the depression of synaptic strengths. Thus, activity levels that would normally result in αCaMKII Thr286 autophosphorylation in the wild-types animals could actually promote phosphatase activity in the mutants. i.e. the mutant phenotype may not only result from the loss of CaMKII function, but also from enhanced phosphatase activities.

One target of PP2B is inhibitor-1, a protein that binds to and inhibits the actions of PP1. The de-phosphorylation of inhibitor-1 releases and thus increases the free levels PP1 (see figure 1.2); an increase in inhibitor-1 would tip the kinase-phosphatase balance even further towards phosphatase activity and synaptic depression. Thus, in addition to the loss of αCaMKII-dependent mechanisms of synaptic potentiation in the mutant mice, mechanisms of synaptic depression could be enhanced and contribute towards the observed enrichment-mediated reduction in mEPSC amplitudes and spine densities.

Another Ca^{2+}/CaM-stimulated protein is nitric oxide synthase (NOS); an increase in NOS activity would increase the production of nitric oxide (NO) and therefore of NO retrograde signaling that mediates activity-dependent increases in presynaptic efficacy (Hawkins et al., 1998). This could have led to the observed decrease in evoked failures and paired-pulse ratios in the enriched mutants which are indicative of increases in release probability.

As CaMKII is also found at high levels within presynaptic axon terminals (Gorelick et al., 1988;Walaas et al., 1989), the presynaptic changes found in the enriched mutant

mice could also have resulted from the loss of Thr286 autophosphorylation directly. One presynaptic target of CaMKII phosphorylation is the N-type Ca^{2+} channel whose phosphorylation has been proposed to impose a negative constraint upon frequency facilitation*; this proposal was the outcome from the observation that frequency facilitation was enhanced in the CA3-specific αCaMKII-knockout mouse, while baseline release probabilities were unaltered (Hinds et al., 2003). CaMKII can also bind to and phosphorylate synapsin I (Benfenati et al., 1992) – a vesicular protein whose phosphorylation-state at a number of sites plays an important role in the mobilisation of synaptic vesicles (e.g. Chi et al., 2003). Interestingly, it was found that LTP-inducing stimulation of Schaffer collaterals in hippocampal 'mini' slices resulted in a long-lasting 6-fold increase in the phosphorylation of synapsin I at its CaMKII sites (Nayak et al., 1996). However, a specific mechanism by which the modulation of synapsin proteins might contribute to the long-term plasticity of neurotransmitter release has not yet been identified (Chi et al., 2003). In fact, mossy fibre to CA3 LTP, as well as Schaffer collateral to CA1 LTP, was found to be intact in hippocampal slices from mice where both synapsin I and II were knocked-out, indicating that synapsin function, and therefore its phosphorylation by CaMKII, can either be fully compensated for by a parallel mechanism or is not necessary for LTP induction or expression (Spillane et al., 1995). In contrast, the activation of presynaptic CaMKII has been proposed to play a crucial role in the activity-dependent un-silencing of synaptic boutons in cultured hippocampal neurones (Ninan and Arancio, 2004).

Another presynaptic protein modulated by its association to αCaMKII is syntaxin 1A. Syntaxin 1A is a key molecule in vesicular trafficking and the SNARE-mediated mechanism of vesicle exocytosis (Jahn and Sudhof, 1999). It has been found that under conditions of increased Ca^{2+} concentration (>1 μM), Thr286 autophosphorylated

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* Frequency facilitation is an activity-dependent increase in evoked release probability that is thought to be brought about by the short-term accumulation of Ca^{2+} within the presynaptic terminal (Zucker and Regehr, 2002).

αCaMKII is the only protein that binds to syntaxin 1A and may therefore be an important element in the regulated process of exocytosis (Ohyama et al., 2002). Ohyama et al (2002) showed that interfering with this CaMKII-syntaxin association decreased the frequency of exocytosis in neurones, indicating that it plays an important role in the regulation of vesicle release. It is not clear, however, how dys-regulation of this process could have resulted in the observed presynaptic phenotype in the enriched mutant mice.

Returning back to the effects of other Ca^{2+}/CaM-stimulated molecules, an increase in activity of the Ca^{2+}/CaM-dependent adenylyl cyclases could have the potential to cause enhanced release probabilities as found in the enriched mutants. Adenylyl cyclases catalyse the production of cAMP which has the capacity to enhance synaptic function through a variety of mechanisms (e.g. Waltereit and Weller, 2003;Ferguson and Storm, 2004). For example, cAMP is the activator of PKA which has numerous pre- and postsynaptic targets (e.g. Nguyen and Woo, 2003), such as RAB3A, a presynaptic vesicle protein that regulates vesicle fusion with the presynaptic membrane and has been found to be essential for the presynaptic form of LTP at mossy fibre synapses in the hippocampus (Castillo et al., 1997). The potential dys-regulation of RAB3A in the mutant mice could, therefore, have contributed towards the altered presynaptic properties of mutant Schaffer collateral axons. Postsynaptic targets of PKA include the GluR1 subunits of AMPA receptors, whose phosphorylation leads to an increase in their membrane expression (Kameyama et al., 1998;Lee et al., 2000;Esteban et al., 2003). However, such increases in postsynaptic efficacy were not detected in the mutants, although such effects could potentially have been masked due to a net depression in postsynaptic efficacy.

Another target of CaM-binding and modulation is the NR1 subunit of the NMDA receptor. Binding has been found to reduce the duration of NMDA receptor channel opening times and charge transfer and, therfore, the influx of Ca^{2+} during synaptic transmission; this may present a form of feedback-inhibition which negatively regulates further Ca^{2+} influx (Ehlers et al., 1996;Rycroft and Gibb, 2002). Under possible enrichment-mediated conditions of increased neuronal activity, Ca^{2+} influx and Ca^{2+}/CaM availability, the loss of CaM-trapping in the mutants could therefore have

resulted in enhanced CaM-dependent feedback-inhibition and reduced NMDA receptor function. This may have resulted in a scenario where levels of NMDA receptor activity, that would normally produce Ca^{2+} signals that initiate mechanisms of synaptic potentiation in wild-type neurones, instead led to Ca^{2+} signals that favoured synaptic depression in the mutant neurones.

In summary, the loss of αCaMKII autonomous activity and the potential dys-regulation of its associations with various pre- and postsynaptic molecules as well as the loss of α-subunit mediated CaM-trapping and therefore potential changes in the activity-dependent stimulation/regulation of other Ca^{2+}/CaM-sensitive proteins, provides a plethora of ways through which the enrichment-mediated changes found in the mutant CA1 neurones could have arisen through aberrant non-wild-type-like plastic processes.

4.6.4 Conclusions

I propose that the enrichment-driven *decreases* in mEPSC median amplitudes and apical dendrite spine densities found in the mutant animals indicate that long-lasting synaptic *depression* occurred in the mutant mouse brains in an experience-dependent manner. Furthermore, I propose that these changes would also have occurred in the wild-type CA1 neurones, but that they were subsequently reversed through αCaMKII Thr286 autophosphorylation-dependent processes (such as LTP). Thus, through the use of environmental enrichment in the $\alpha CaMKII^{T286A}$ mutant mouse I have uncovered evidence of physiological plasticity that may occur during the processing of memory in the mouse brain. This interpretation is consistent with two widely-held hypotheses. The first is that synaptic depression is equally as important as synaptic potentiation for learning and memory (Braunewell and Manahan-Vaughan, 2001). The second is that plastic changes that occur in the hippocampus during cognitive processing are only temporary and that this brain structure is not the primary site for the long-term storage of memory traces (e.g. Squire, 1992).

I surmised that any enrichment-mediated changes would be observed in the CA1 cells from wild-type mice and that such changes would be blocked in the mutants due to the impairment of αCaMKII-dependent plastic processes (Giese et al., 1998). This was not

the case as enrichment-mediated changes were found in both the wild-type and mutant neurones.

In the mutant animals, EPSC1 amplitudes were increased whilst the probability of failures and PPR of evoked currents, mEPSC median amplitudes and apical dendrite spine densities decreased. In the wild-type neurones, however, the only effect of enrichment detected was to the distribution of median mEPSC amplitudes across recordings. The observed changes in the evoked currents in the enriched mutant mice are indicative of increases in presynaptic release probability. I propose that this does not reflect a primary effect of enrichment that fails to be subsequently reversed, but that it is more likely to be a compensatory change that occurs in the enriched mutants in order to increase excitatory drive and maintain the level of excitatory CA1 input within an optimal window. Thus, these effects are only seen in the mutant mice with no similar trends in their wild-type littermates.

In contrast, the decreases in mEPSC amplitudes and apical spine densities were not found to reach statistically significant levels in the wild-type mice, whereas trends in the same direction were found for both of these measurements. These observations support the conclusion that similar enrichment-driven changes would have occurred in the wild-type mice, but that they were indeed actively reversed in a Thr286 autophosphorylation-dependent manner.

In conclusion, I consider that these identified changes seen in the enriched mutant mice are real enrichment-driven modifications that reveal new and relevant information about behaviourally-induced hippocampal synaptic plasticity in mice.

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